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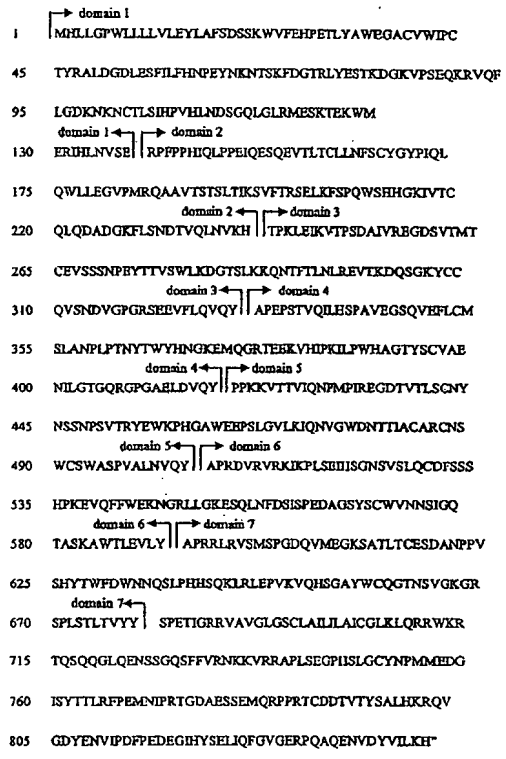

(57) Abstract: The invention concerns treatment methods using anti-CD22 monoclonal antibodies with unique physiologic properties. In particular, the invention concerns methods for the treatment of B-cell malignancies by administering an effective amount of a blocking anti-CD22 monoclonal antibody specifically binding to the first two Ig-like domains, or to an epitope within the first two Ig-like domains of native human CD22 (hCD22).

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TREATMENT METHODS USING ANTI-CD22 ANTIBODIES

Background of the Invention

Field of the Invention

[0002] The present invention concerns the therapeutic use of certain anti-CD22 monoclonal antibodies with unique physiologic properties. More specifically, the invention concerns methods of treating B-cell malignancies, such as lymphomas and leukemias, and autoimmune diseases with blocking anti-CD22 antibodies having unique pro-apoptotic properties.

Description of the Related Art

[0003] CD22 is a membrane glycophosphoprotein found on nearly all B lymphocytes and most B-cell lymphomas. Cross-linking CD22 triggers CD22 tyrosine phosphorylation and assembles a complex of effector proteins that activate the stress-activated protein kinase (SAPK) pathway. CD22 cross-linking provides a potent costimulatory signal in primary B-cells and pro-apoptotic signal in neoplastic B-cells. Structurally, CD22 is a member of the "sialoadhesin" subclass of the immunoglobulin (Ig) gene superfamily, having seven extracellular Ig domains with a single amino-terminal V-set Ig domain and six C-2 set Ig domains. Wilson *et al., J. Exp. Med.* 173:137-146 (1991); Engel *et al., J. Exp. Med.* 181:1581-1586 (1995); and Torres *et al., J. Immunol.* 149:2641-2649 (1992). It has been shown that CD22 is a critical lymphocyte-specific signal transduction molecule which negatively and positively regulates B lymphocyte antigen receptor (BCR) signaling by recruiting signaling effector molecules to physiologically pertinent sites. Tedder *et al., Annu. Rev. Immunol.* 15:481-504 (1997); Sato *et al., Immunology* 10:287-297 (1998).

[0004] Anti-CD22 antibodies have been described, for example in U.S. Patent Nos. 5,484,892; 6,183,744; 6,187,287; 6,254,868, and in Tuscano *et al., Blood* 94(4):1382-92 (1999). The use of monoclonal antibodies, including anti-CD22 antibodies, in the treatment of non-Hodgkin's lymphoma is reviewed, for example, by Renner *et al., Leukemia* 11(Suppl. 2):S55-9 (1997). A humanized anti-CD22 antibody, LymphoCide™ (empatuzumab, Immunomedics, Inc.) is in Phase III clinical trials for the treatment of indolent and aggressive forms of non-Hodgkin's lymphomas. An yttrium-90-labeled version of this antibody is currently in Phase I clinical trials for the same indication.

[0005] Despite recent advances in cancer therapy, B-cell malignancies, such as the B-cell subtype of non-Hodgkin's lymphoma, and chronic lymphocytic leukemia, are major

contributors of cancer-related deaths. Accordingly, there is a great need for further, improved therapeutic regimens for the treatment of B-cell malignancies.

Summary of the Invention

[0006] The present invention concerns an improved clinical approach for the treatment of B-cell malignancies in human patients, taking advantage of the unique properties of certain blocking anti-CD22 monoclonal antibodies.

[0007] In one aspect, the invention concerns a method for treating a human patient diagnosed with a B-cell malignancy, comprising (1) administering to the patient an effective amount of a blocking anti-CD22 monoclonal antibody specifically binding to the first two Ig-like domains or to an epitope associated with the first two Ig-like domains of native human CD22 (hCD22) of SEQ ID NO: 1, and (2) monitoring the response of the malignancy to the treatment.

[0008] In a particular embodiment, the antibody used binds to essentially the same epitope as an antibody selected from the group consisting of HB22-7 (HB11347), HB22-23 (HB11349), HB22-33, HB22-5, HB22-13, and HB22-196, preferably HB22-7, HB22-23, or HB22-33, more preferably HB22-7 or HB22-33.

[0009] In a further embodiment, the antibody blocks CD22 binding to its ligand by at least 70%, preferably by at least 80%.

[0010] In another embodiment, the antibody comprises a heavy chain comprising a V_H sequence having at least about 95 % sequence identity with the sequence of amino acids 1 to 100 of SEQ ID NO: 9 (HB22-5 V_H sequence); or amino acids 1 to 97 of SEQ ID NO: 11 (HB22-7 V_H sequence); or amino acids 1 to 100 of SEQ ID NO: 13 (HB22-13 V_H sequence); or amino acids 1 to 100 of SEQ ID NO: 15 (HB22-23 V_H sequence); or amino acids 1 to 98 of SEQ ID NO: 17 (HB22-33 V_H sequence); or amino acids 1 to 100 of SEQ ID NO: 19 (HB22-196 V_H sequence).

[0011] In yet another embodiment, the antibody comprises a heavy chain comprising a V_H sequence having at least about 95 % sequence identity with the sequence of amino acids 1 to 97 of SEQ ID NO: 11 (HB22-7 V_H sequence); or amino acids 1 to 100 of SEQ ID NO: 15 (HB22-23 V_H sequence); or amino acids 1 to 98 of SEQ ID NO: 17 (HB22-33 V_H sequence).

[0012] In a still further embodiment, the antibody comprises a V_H sequence selected from the group consisting of amino acids 1 to 97 of SEQ ID NO: 11 (HB22-7 V_H sequence); amino acids 1 to 100 of SEQ ID NO: 15 (HB22-23 V_H sequence); and amino acids 1 to 98 of SEQ ID NO: 17 (HB22-33 V_H sequence).

[0013] In a different embodiment, the antibody comprises a light chain comprising a V_κ sequence having at least about 95 % sequence identity with the amino acid sequence of SEQ ID NO: 21 (HB22-5 V_κ sequence); or SEQ ID NO: 23 (HB22-7 V_κ sequence); or SEQ ID NO: 25

(HB22-13 V_κ sequence); or SEQ ID NO: 27 (HB22-23 V_κ sequence); or SEQ ID NO: 29 (HB22-33 V_κ sequence); or SEQ ID NO: 31 (HB22-196 V_κ sequence).

[0014] In a particular embodiment, the antibody comprises a light chain comprising a V_κ sequence having at least about 95 % sequence identity with the amino acid sequence of SEQ ID NO: 23 (HB22-7 V_κ sequence); or SEQ ID NO: 27 (HB22-23 V_κ sequence); or SEQ ID NO: 29 (HB22-33 V_κ sequence).

[0015] In a further embodiment, the antibody comprises a V_κ sequence selected from the group consisting of the amino acid sequence of SEQ ID NO: 23 (HB22-7 V_κ sequence); SEQ ID NO: 27 (HB22-23 V_κ sequence); and SEQ ID NO: 29 (HB22-33 V_κ sequence).

[0016] In a preferred embodiment, the antibody comprises V_H and V_κ sequences selected from the group consisting of amino acids 1 to 97 of SEQ ID NO: 11 (HB22-7 V_H sequence) and the amino acid sequence of SEQ ID NO: 23 (HB22-7 V_κ sequence); amino acids 1 to 100 of SEQ ID NO: 15 (HB22-23 V_H sequence) and the amino acid sequence of SEQ ID NO: 27 (HB22-23 V_κ sequence); and amino acids 1 to 98 of SEQ ID NO: 17 (HB22-33 V_H sequence) and the amino acid sequence of SEQ ID NO: 29 (HB22-33 V_κ sequence).

[0017] In a different aspect, the invention concerns nucleic acid encoding any of the antibody heavy or light chain variable regions discussed above, or any portion thereof.

[0018] The targeted condition can be any type of B-cell malignancy, including but not limited to localized B-cell malignancies. Typical representatives of B-cell malignancies are B-cell subtype of non-Hodgkin's lymphoma, Burkitt's lymphoma, multiple myeloma, chronic lymphocytic leukemia, hairy cell leukemia, and prolymphocytic leukemia.

[0019] The treatment method of the present invention may be performed without any further treatment of malignant B cells, including radiation therapy, chemotherapy, combined modality radioimmunotherapy (CMRIT), and the like. The treatment method of the present invention typically provides improved cure rate and/or increased survival and/or superior tumor volume reduction when compared to no treatment, combination treatment with the same antibody and radioimmunotherapy, or with radioimmunotherapy alone.

[0020] The antibody can be a complete antibody, or an antibody fragment, including, for example, Fab, Fab', $F(ab')_2$, and Fv fragments, diabodies, linear antibodies, single-chain antibody molecules, and multispecific antibodies formed from antibody fragments. Thus, the antibody may have an additional antigen specificity, e.g. may be a bispecific antibody. The bispecific antibody may, for example, additionally bind to another epitope to CD22. In addition,

the bispecific antibody may have binding specificity for other antigens, such as, CD19, CD20, CD52, CD3, CD28, or HLA-DR10 (Lym-1); or for Fc receptors, e.g. CD16, CD64 and CD89.

[0021] The antibody may be chimeric, humanized, primatized, or human.

[0022] The administration of the antibody may be performed by any conventional route, such as intravenous (i.v.) administration by repeated intravenous infusions.

[0023] The response to the treatment may be monitored by methods well known for a skilled practitioner, including monitoring shrinkage of a solid tumor, e.g. by magnetic resonance imaging (MRI).

Brief Description of the Drawings

[0024] Figure 1 shows the amino acid sequence of human CD22 (hCD22), where the boundaries of the Ig-like domains (domains 1-7) are indicated

[0025] Figure 2. Whole body autoradiography of Raji and Ramos tumor-bearing nude mice injected with ^{111}In-2IT-BAD-antiCD22 (HB22-7). Mice were sacrificed and autoradiographed 48 hours after injection. Upper image is Raji-tumored mouse, lower image is Ramos-tumored mouse.

[0026] Figure 3. The temporal assessment of tumor volume in Raji-xenografted mice that were untreated or treated with 125 uCi ^{90}Y-DOTA-peptide-Lym-1 (RIT) alone, anti-CD22 alone (HB22-7), or three different sequences of RIT and HB22-7 (CMRIT) in trial 081500. Tumor volume was assessed three times per week. Mouse numbers for each treatment group are tabulated (Table 2).

[0027] Figure 4. Summary analysis of tumor volume observed in all independent xenograft trials. The trials were conducted as described in Figure 2. Mouse numbers for each trail are tabulated (Table 2).

[0028] Figure 5. The response and cure rate for Raji-xenografted mice that were treated as described in Figure 2. The tumor responses were categorized as follows: C, cure (tumor disappeared and did not regrow by the end of the 84-day study); CR, complete regression (tumor disappeared for at least 7 days but later regrew); PR, partial regression (tumor volume decreased by 50% or more for at least 7 days, then regrew). The data represents results of all independent trials.

[0029] Figure 6. Overall survival was assessed for Raji xenografted mice that were treated as described in Figure 2. Mice were euthanized when the tumor burden exceeded 2000 mg or at the end of the 84 day trial. The data represents results of all independent trials.

[0030] Figures 7a, 7b and 7c. Hematologic toxicity was assessed by measuring white blood cell (WBC) (Figure 7b), red blood cell (RBC) (Figure 7c) and platelet counts (Figure 7a)

twice weekly in the Raji-xenografted mice that were treated as described in Figure 2. When compared to RIT alone there was no difference in hematologic toxicity in the CMRIT groups. In addition, there was no hematologic toxicity observed in the mice treated with HB22-7 alone.

[0031] Figure 8. Non-hematologic toxicity was assessed by measuring body weights twice weekly in Raji xenografted mice that were treated as described in Figure 2. There were no significant differences in body weights in any of the treatment groups in all five xenograft trials.

[0032] Figure 9. RIT clearance was assessed by measuring radioactivity in whole body (WB) and blood daily for 5 days after initiation of treatment with RIT. The results were reported after adjusting for decay based on the $T_{1/2}$ of ^{90}Y. There were no significant differences in RIT clearance in any of the CMRIT treatment groups.

[0033] Figure 10. V_H amino acid sequence analysis of anti-CD22 antibodies (Abs) that block ligand binding. Amino acid numbering and designations of the origins of the coding sequence for each Ab is according to the convention of Kabat *et al.* (*Sequences of Proteins of Immunological Interest*, U.S. Government Printing Office, Bethesda, MD, 1991), where amino acid positions 1-94, CDR1 and 2, and FR1, 2, and 3 are encoded by a V_H gene. Sequences that overlap with the 5' PCR primers are not shown. A dot indicates a gap inserted in the sequence to maximize alignment of similar amino acid sequences. Gaps in the sequences were introduced between V_H, D and J segments for clarity. The rank order of sequences shown was based on relatedness to the HB22-5 sequence.

[0034] Figures 11-16. Nucleotide and encoded amino acid sequences for heavy chain V_H-D-J_H junctional sequences for anti-CD22 Abs from hybridomas HB22-5 (SEQ ID NOS: 8 and 9), HB22-7 (SEQ ID NOS: 10 and 11); HB22-13 (SEQ ID NOS: 12 and 13); HB22-23 (SEQ ID NOS: 14 and 15); HB22-33 (SEQ ID NOS: 16 and 17); and HB22-196 (SEQ ID NOS: 18 and 19). Sequences that overlap with the 5' PCR primers are indicated by double underlining. D region sequences are underlined.

[0035] Figure 17. Light chain Vκ amino acid sequence analysis of anti-CD22 Abs that block ligand binding. Amino acid numbering and designation of origins of the coding sequence for each Ab is according to the convention of Kabat *et al., supra.* The amino acid following the predicted signal sequence cleavage site is numbered 1. A dot indicates a gap inserted in the sequence to maximize alignment of similar amino acid sequences. Gaps in the sequences were introduced between Vκ, J segments and κ constant region (double underlined) sequences for clarity.

[0036] Figures 18-23. Nucleotide and deduced amino acid sequences for kappa light chain V-J-constant region junctional sequences for anti-CD22 Abs from hybridomas HB22-5

(SEQ ID NOS: 20 and 21); HB22-7 (SEQ ID NOS: 22 and 23): HB22-13 (SEQ ID NOS: 24 and 25): HB22-23 (SEQ ID NOS: 26 and 27); HB22-33 (SEQ ID NOS: 28 and 29); and HB22-196 (SEQ ID NOS: 30 and 31). Sequences that overlap with the 5' PCR primers are indicated by double underlining.

Detailed Description of the Preferred Embodiments

A. Definitions

[0037] Unless defined otherwise, technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

[0038] One skilled in the art will recognize many methods and materials similar or equivalent to those described herein, which could be used in the practice of the present invention. Indeed, the present invention is in no way limited to the methods and materials described. For purposes of the present invention, the following terms are defined below.

[0039] The term "immunoglobulin" (Ig) is used to refer to the immunity-conferring portion of the globulin proteins of serum, and to other glycoproteins, which may not occur in nature but have the same functional characteristics. The term "immunoglobulin" or "Ig" specifically includes "antibodies" (Abs). While antibodies exhibit binding specificity to a specific antigen, immunoglobulins include both antibodies and other antibody-like molecules that lack antigen specificity. Native immunoglobulins are secreted by differentiated B cells termed plasma cells, and immunoglobulins without any antigen specificity are produced at low levels by the lymph system and at increased levels by myelomas. As used herein, the terms "immunoglobulin," "Ig," and grammatical variants thereof are used to include antibodies (as hereinabove defined), and Ig molecules without antigen specificity.

[0040] Native immunoglobulins are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies among the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain at one end (V_L) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light- chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light- and heavy-chain variable domains.

[0041] The main Ig isotypes (classes) found in serum, and the corresponding Ig heavy chains, shown in parentheses, are listed below:

[0042] IgG (γ chain): the principal Ig in serum, the main antibody raised in response to an antigen, this antibody crosses the placenta;

[0043] IgE (ε chain): this Ig binds tightly to mast cells and basophils, and when additionally bound to antigen, causes release of histamine and other mediators of immediate hypersensitivity; plays a primary role in allergic reactions, including hay fever, asthma and anaphylaxis; and may serve a protective role against parasites;

[0044] IgA (α chain): this Ig is present in external secretions, such as saliva, tears, mucous, and colostrum;

[0045] IgM (μ chain): the Ig first induced in response to an antigen; it typically has lower affinity than other antibody isotypes produced later and is typically pentameric.

[0046] IgD (δ chain): this Ig is found in relatively high concentrations in umbilical cord blood, may be an early cell receptor for antigen, and is the main lymphocyte cell surface molecule.

[0047] The term "antibody" herein is used in the broadest sense and specifically covers monoclonal antibodies (including, but not limited to, full length monoclonal antibodies), polyclonal antibodies, multispecific antibodies (*e.g.*, bispecific antibodies), and antibody fragments so long as they exhibit the desired biological activity.

[0048] "Antibody fragments" comprise a portion of a full length antibody, generally the antigen binding or variable (V) domain. Examples of antibody fragments include Fab, Fab', $F(ab')_2$, and Fv fragments; diabodies; linear antibodies; single-chain antibody molecules; and multispecific antibodies formed from antibody fragments.

[0049] The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen.

[0050] The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins), as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Patent No. 4,816,567; Morrison *et al., Proc. Natl. Acad. Sci. USA*

81:6851-6855 (1984); Oi *et al., Biotechologies* 4(3):214-221 (1986); and Liu *et al., Proc. Natl. Acad Sci. USA* 84:3439-43 (1987)).

[0051] "Humanized" or "CDR grafted" forms of non-human (*e.g.*, murine) antibodies are human immunoglobulins (recipient antibody) in which hypervariable region residues of the recipient are replaced by hypervariable region residues from a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, framework region (FR) residues of the human immunoglobulin are also replaced by corresponding non-human residues (so called "back mutations"). Furthermore, humanized antibodies may be modified to comprise residues which are not found in the recipient antibody or in the donor antibody, in order to further improve antibody properties, such as affinity. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable regions correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones *et al., Nature* 321:522-525 (1986); and Reichmann *et al., Nature* 332:323-329 (1988).

[0052] "Single-chain Fv" or "sFv" antibody fragments comprise the V_H and V_L domains of antibody, wherein these domains are present in a single polypeptide chain. Generally, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the sFv to form the desired structure for antigen binding. For a review of sFv see Plückthun in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenburg and Moore eds. Springer-Verlag, New York, pp. 269-315 (1994).

[0053] The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy chain variable domain (V_H) connected to a light chain variable domain (V_L) in the same polypeptide chain (V_H - V_L). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger *et al., Proc. Natl. Acad. Sci. USA* 90:6444-6448 (1993).

[0054] The expression "linear antibodies" when used throughout this application refers to the antibodies described in Zapata *et al. Protein Eng.* 8(10):1057-1062 (1995). Briefly, these antibodies comprise a pair of tandem Fd segments (V_H-C_H1-V_H-C_H1) which form a pair of antigen binding regions. Linear antibodies can be bispecific or monospecific.

[0055] Antibodies of the IgG, IgE, IgA, IgM, and IgD isotypes may have the same variable regions, i.e. the same antigen binding cavities, even though they differ in the constant region of their heavy chains. The constant regions of an immunoglobulin, e.g. antibody are not involved directly in binding the antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody-dependent cellular toxicity (ADCC).

[0056] Some of the main antibody isotypes (classes) are divided into further sub-classes. IgG has four known subclasses: IgG1 (γ1), IgG2 (γ2), IgG3 (γ3), and IgG4 (γ4), while IgA has two known sub-classes: IgA1 (α1) and IgA2 (α2).

[0057] The term "epitope" is used to refer to binding sites for (monoclonal or polyclonal) antibodies on protein antigens.

[0058] Antibodies which bind to domain 1 and/or 2 within the amino acid sequence of native sequence human CD22, or to essentially the same epitope(s) bound by any of monoclonal antibodies specifically disclosed herein, such as HB22-7, HB22-23, and HB22-33, can be identified by "epitope mapping." There are many methods known in the art for mapping and characterizing the location of epitopes on proteins, including solving the crystal structure of an antibody-antigen complex, competition assays, gene fragment expression assays, and synthetic peptide-based assays, as described, for example, in Chapter 11 of Harlow and Lane, Using Antibodies, a Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1999. According to the gene fragment expression assays, the open reading frame encoding the protein is fragmented either randomly or by specific genetic constructions and the reactivity of the expressed fragments of the protein with the antibody to be tested is determined. The gene fragments may, for example, be produced by PCR and then transcribed and translated into protein *in vitro*, in the presence of radioactive amino acids. The binding of the antibody to the radioactively labeled protein fragments is then determined by immunoprecipitation and gel electrophoresis. Certain epitopes can also be identified by using large libraries of random peptide sequences displayed on the surface of phage particles (phage libraries). Alternatively, a defined library of overlapping peptide fragments can be tested for binding to the test antibody in simple binding assays. The latter approach is suitable to define linear epitopes of about 5 to 15 amino acids.

[0059] An antibody binds "essentially the same epitope" as a reference antibody, when the two antibodies recognize identical or sterically overlapping epitopes. The most widely used and rapid methods for determining whether two epitopes bind to identical or sterically overlapping epitopes are competition assays (e.g. competition ELISA assays), which can be configured in all number of different formats, using either labeled antigen or labeled antibody. Usually, the antigen

is immobilized on a 96-well plate, and the ability of unlabeled antibodies to block the binding of labeled antibodies is measured using radioactive or enzyme labels.

[0060] The term amino acid or amino acid residue, as used herein, refers to naturally occurring L amino acids or to D amino acids as described further below with respect to variants. The commonly used one- and three-letter abbreviations for amino acids are used herein (Bruce Alberts et al., Molecular Biology of the Cell, Garland Publishing, Inc., New York (3d ed. 1994)).

[0061] "Sequence identity" is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in a native polypeptide sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. The % sequence identity values are generated by the NCBI BLAST2.0 software as defined by Altschul *et al.*, (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res., 25:3389-3402. The parameters are set to default values, with the exception of the Penalty for mismatch, which is set to –1.

[0062] As used herein, "treatment" is an approach for obtaining beneficial or desired clinical results. For purposes of this invention, beneficial or desired clinical results include, but are not limited to, alleviation of symptoms, diminishment of extent of disease, stabilized (i.e., not worsening) state of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, and remission (whether partial or total), whether detectable or undetectable. "Treatment" can also mean prolonging survival as compared to expected survival if not receiving treatment. "Treatment" is an intervention performed with the intention of preventing the development or altering the pathology of a disorder. Accordingly, "treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented. In the context of B cell malignancies, the treatment may reduce the number of malignant cells; reduce the tumor size; inhibit (slow down or stop) the spread of malignant cells, including infiltration into peripheral organs, e.g. soft tissue or bone; inhibit (slow down or stop) metastasis; inhibit tumor growth; provide relief from symptoms associated with a B cell malignancy; reduce mortality; improve quality of life, etc. Treatment with the antibodies herein may result in cytostatic and/or cytotoxic effects.

[0063] The term "B cell malignancy," and grammatical variants thereof, are used in the broadest sense to refer to malignancies or neoplasms of B cells that typically arise in lymphoid tissues, such as bone marrow or lymph nodes, but may also arise in non-lymphoid tissues, such as

thyroid, gastrointestinal tract, salivary gland and conjunctiva. The treatment methods of the present invention specifically concern CD22-positive B cell malignancies including, without limitation, B-cell subtype of non-Hodgkin's lymphoma, Burkitt's lymphoma, multiple myeloma, chronic lymphocytic leukemia, hairy cell leukemia, and prolymphocytic leukemia.

B. Detailed Description

1. Antibodies

[0064] Blocking anti-CD22 monoclonal antibodies designated HB22-7, HB22-23, HB22-33, HB22-5, HB22-13, and HB22-196 are known, and have been disclosed in U.S. Patent No. 5,484,892, Tuscano *et al., Eur. J. Immunol.* 26:1246 (1996), and Tuscano *et al., Blood* 94(4), 1382-1392 (1999). HB22-7 and HB22-23 are available from the American Type Culture Collection (ATCC), 12302 Parklawn Drive, Rockville, Md. 20852, under Accession Nos. HB22347 and HB11349, respectively. The preparation of these antibodies is also described in Example 1 below. Epitope mapping of CD22 has shown that these blocking monoclonal antibodies bind to the first two Ig-like domain or to epitopes which are associated with the first two Ig-like domain of human CD22 (U.S. Patent No. 5,484,892 and Tedder *et al., Annu. Rev. Immunol.* 15:481-504 (1997)). The heavy and light chain variable region sequences of the antibodies are also disclosed in the present application.

[0065] The present invention is based on the unexpectedly superior properties of blocking anti-CD22 antibodies having the overall characteristics of HB22-7, HB22-23, HB22-33, HB22-5, HB22-13, and HB22-196 in the treatment of B-cell malignancies, based on results obtained in a xenograft model of B-cell type non-Hodgkin's lymphoma (NHL).

[0066] The anti-CD22 monoclonal antibodies can be made by any standard method known in the art, such as, for example, by the hybridoma method (Koehler and Milstein, *Nature* 256:495-497 (1975); and Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103, (Academic Press, 1986)), or by recombinant techniques, disclosed, for example, in U.S. Patent No. 4,816,567, and by Wood *et al., Nature* 314:446-9 (1985).

[0067] It is now also possible to produce transgenic animals (e.g. mice) that are capable, upon immunization, of producing a repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production

of human antibodies upon antigen challenge. See, e.g. Jakobovits *et al.*, *Proc. Natl. Acad. Sci. USA* 90, 2551-255 (1993); Jakobovits *et al.*, *Nature* 362, 255-258 (1993).

[0068] Mendez *et al.* (*Nature Genetics* 15: 146-156 (1997)) have further improved the technology and have generated a line of transgenic mice designated as "Xenomouse II" that, when challenged with an antigen, generates high affinity fully human antibodies. This was achieved by germ-line integration of megabase human heavy chain and light chain loci into mice with deletion into endogenous J_H segment as described above. The Xenomouse II harbors 1,020 kb of human heavy chain locus containing approximately 66 V_H genes, complete D_H and J_H regions and three different constant regions (μ, δ and χ), and also harbors 800 kb of human κ locus containing 32 Vκ genes, Jκ segments and Cκ genes. The antibodies produced in these mice closely resemble that seen in humans in all respects, including gene rearrangement, assembly, and repertoire. The human antibodies are preferentially expressed over endogenous antibodies due to deletion in endogenous J_H segment that prevents gene rearrangement in the murine locus.

[0069] Alternatively, the phage display technology (McCafferty *et al.*, *Nature* 348, 552-553 (1990)) can be used to produce human antibodies and antibody fragments *in vitro*, from immunoglobulin variable (V) domain gene repertoires from unimmunized donors. According to this technique, antibody V domain genes are cloned in-frame into either a major or minor coat protein gene of a filamentous bacteriophage, such as M13 or fd, and displayed as functional antibody fragments on the surface of the phage particle. Because the filamentous particle contains a single-stranded DNA copy of the phage genome, selections based on the functional properties of the antibody also result in selection of the gene encoding the antibody exhibiting those properties. Thus, the phage mimics some of the properties of the B-cell. Phage display can be performed in a variety of formats; for their review see, e.g. Johnson, Kevin S. and Chiswell, David J., *Current Opinion in Structural Biology* 3, 564-571 (1993). Several sources of V-gene segments can be used for phage display. Clackson *et al.*, *Nature* 352, 624-628 (1991) isolated a diverse array of anti-oxazolone antibodies from a small random combinatorial library of V-genes derived from the spleens of immunized mice. A repertoire of V-genes from unimmunized human donors can be constructed and antibodies to a diverse array of antigens (including self-antigens) can be isolated essentially following the techniques described by Marks *et al.*, *J. Mol. Biol.* 222, 581-597 (1991), or Griffith *et al.*, *EMBO J.* 12, 725-734 (1993). In a natural immune response, antibody genes accumulate mutations at a high rate (somatic hypermutation). Some of the changes introduced will confer higher affinity, and B cells displaying high-affinity surface immunoglobulin are preferentially replicated and differentiated during subsequent antigen challenge. This natural process can be mimicked by employing the technique known as "chain shuffling" (Marks *et al.*,

Bio/Technol. 10, 779-783 [1992]). In this method, the affinity of "primary" human antibodies obtained by phage display can be improved by sequentially replacing the heavy and light chain V-region genes with repertoires of naturally occurring variants (repertoires) of V-domain genes obtained from unimmunized donors. This techniques allows the production of antibodies and antibody fragments with affinities in the nM range. A strategy for making very large phage antibody repertoires has been described by Waterhouse *et al.*, *Nucl. Acids Res.* 21, 2265-2266 (1993).

[0070] For further information concerning the production of monoclonal antibodies see also Goding, J.W., Monoclonal Antibodies: Principles and Practice, 3rd Edition, Academic Press, Inc., London, San Diego, 1996; Liddell and Weeks: Antibody Technology: A Comprehensive Overview, Bios Scientific Publishers: Oxford, UK, 1995; Breitling and Dubel: Recombinant Antibodies, John Wiley & Sons, New York, 1999; and Phage Display: A Laboratory Manual, Barbas *et al.*, editors, Cold Springs Harbor Laboratory, Cold Spring Harbor, 2001.

[0071] Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, *e.g.*, Morimoto *et al.*, *J. Biochem. Biophys. Methods* 24:107-117 (1992) and Brennan *et al.*, *Science* 229:81 (1985)). However, these fragments can now be produced directly by recombinant host cells. For example, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form $F(ab')_2$ fragments (Carter *et al.*, *Bio/Technology* 10:163-167 (1992)). In another embodiment, the $F(ab')_2$ is formed using the leucine zipper GCN4 to promote assembly of the $F(ab')_2$ molecule. According to another approach, Fv, Fab or $F(ab')_2$ fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner.

[0072] Heteroconjugate antibodies, composed of two covalently joined antibodies, are also within the scope of the present invention. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (U.S. Patent No. 4,676,980), and for treatment of HIV infection (PCT application publication Nos. WO 91/00360 and WO 92/200373). Heteroconjugate antibodies may be made using any convenient cross-linking methods, using well known, commercially available cross-linking agents.

[0073] The antibodies of the present invention, whether rodent, human, or humanized may also have a further antigen-specificity, to form bispecific antibodies. The second binding specificity may be directed, for example, against a further B cell antigen, such as CD19, CD20, CD52, and other CD antigens expressed on B cells, especially antigens associated with the targeted B cell malignancy. For example, CD20 is known to be expressed in more than 90% of

non-Hodgkin's lymphomas. An anti-CD20 antibody (Rituxan®, IDEC Pharmaceuticals) is in clinical use for the treatment of non-Hodgkin's lymphoma. CAMPATH-1H (anti-CD52w) is another antibody developed for treating B cell malignancies. Bispecific antibodies including a binding specificity to the CD20 or CD52 antigen are specifically included within the scope herein. Another B cell antigen to which the bispecific antibodies of the present invention can bind is HLA-DR10 (Lym-1), a known marker of non-Hodgkin's lymphoma. Bispecific antibodies can be generated to enhance tumor localization as well as to recruit and/or augment the tumor-specific immune response. Examples of other antigen targets include, CD3, CD28, and the Fc receptors (CD16, CD64 and CD89). Bispecific antibodies are expected to have enhanced cytotoxicity and, as a result, improved remission rate and survival.

[0074] Antibodies binding to essentially the same epitope as HB22-7, HB22-23, HB22-33, HB22-5, HB22-13, and/or HB22-196 can be identified by epitope mapping. The simplest way to determine whether two different antibodies recognize the same epitope is a competition binding assay. This method determines if the antibodies are able to block each other's binding to the antigen, and works for both conformational and linear epitopes. The competition binding assay can be configured in a large number of different formats using either labeled antigen or labeled antibody. In the most common version of this assay, the antigen is immobilized on a 96-well plate. The ability of unlabeled antibodies to block the binding of labeled antibodies to the antigen is then measured using radioactive or enzyme labels. For further details see, for example, Wagener *et al., J. Immunol.*, 130:2308-2315 (1983); Wagener *et al., J. Immunol. Methods,* 68:269-274 (1984); Kuroki *et al., Cancer Res.* 50:4872-4879 (1990); Kuroki *et al., Immunol. Invest.* 21:523-538 (1992); Kuroki *et al., Hybridoma* 11:391-407 (1992), and Using Antibodies: A Laboratory Manual, Ed Harlow and David Lane editors, Cold Springs Harbor Laboratory Press, Cold Springs Harbor, New York, 1999, pp. 386-389.

[0075] Alternatively, or in addition, epitope mapping can be preformed by using a technique based on fragmentation of the antigen to which the antibody binds, either randomly or by specific genetic construction, and determining the reactivity of the fragments obtained with the antibody. Fragmentation can also be performed on the nucleic acid level, for example by PCR technique, followed by transcription and translation into protein *in vitro* in the presence of radioactive amino acids. For further details see, for example, Harlow and Lane, *supra*, pp. 390-392.

[0076] According to a further method of epitope mapping, a set of overlapping peptides is synthesized, each corresponding to a small linear segment of the protein antigen, and arrayed on a solid phase. The panel of peptides is then probed with the test antibody, and bound

antibody is detected using an enzyme-labeled secondary antibody. (Harlow and Lane, *supra*, pp. 393-396.)

[0077] An additional method well known in the art for epitope mapping is antibody selection from random synthetic or phage display peptide library. Phage display libraries are constructed by cloning complex mixtures of peptide-encoding oligonucleotides into the amino terminus of the minor coat protein gene of the f1-type ssDNA phage. Such phage display libraries are commercially available, for example, from New England Biolabs. The libraries are amplified as stocks, and then an aliquot sufficient to represent multiple copies of each independent clone is mixed with the antibody of interest. Antibody-bound phage are collected by a procedure called "biopanning," and unbound phage are removed. The bound phage are eluted and used to infect bacteria, and the selected stock is amplified. Individual plaques of the final selected stock are growth and checked for specific antibody reactivity, e.g. by ELISA, and the DNA around the insert site is sequenced. Analysis of the sequence encoding the peptide to which the antibody binds defined the specificity of the antibody. For further details see, e.g. Smith and Scott, *Methods Enzymol.* 217:228-257 (1993), and Harlow and Lane, *supra*, pp. 397-398.

[0078] Non-human (rodent) antibodies can be further modified, to make them more suitable for human clinical application. Chimeric antibodies are produced with mouse variable region gene segments of desired specificity spliced into human constant domain gene segments (see, e.g. U.S. Patent No. 4,816,567).

[0079] Non-human (rodent) antibodies can also be humanized, in order to avoid issues of antigenicity when using the antibodies in human therapy. Generally, a humanized antibody has one or more amino acid residues introduced into it from a non-human source. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones et al., *Nature*, 321:522-525 (1986); Riechmann et al., *Nature*, 332:323-327 (1988); Verhoeyen et al., *Science*, 239:1534-1536 (1988)), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Despite the relatively straightforward nature of antibody humanization, simple grafting of the rodent CDR's into human frameworks (FR) does not always reconstitute the binding affinity and specificity of the original rodent monoclonal antibody. Properties of a humanized antibody can be improved by suitable design, including, for example, substitution of residues from the rodent antibody into the human framework (backmutations). The positions for such backmutations can be determined by sequence and structural analysis, or by analysis of the variable regions' three-dimensional model. In addition, phage display libraries can be used to vary amino acids at chosen positions within the

antibody sequence. The properties of a humanized antibody are also affected by the choice of the human framework. Early experiments used a limited subset of well-characterized human monoclonal antibodies, irrespective of the sequence identity to the rodent monoclonal antibody (the so-called fixed frameworks approach). More recently, some groups use variable regions with high amino acid sequence identity to the rodent variable regions (homology matching or best-fit method). According to another approach, consensus or germline sequences are used, or fragments of the framework sequences within each light or heavy chain variable region are selected from several different human monoclonal antibodies.

[0080] Amino acid variants of antibodies prepared by any technique discussed above or otherwise available can be prepared by introducing appropriate nucleotide changes into the anti-CD22 DNA, or, for example, by peptide synthesis. The amino acid changes also may alter post-translational processes of the humanized or variant anti-CD22 antibody, such as changing the number or position of glycosylation sites.

[0081] Antibodies are glycosylated at conserved positions in their constant regions (Jefferis and Lund, *Chem. Immunol.* 65:111-128 (1997); Wright and Morrison, *TibTECH* 15:26-32 (1997)). The oligosaccharide side chains of the immunoglobulins affect the protein's function (Boyd *et al., Mol. Immunol.* 32:1311-1318 (1996); Wittwe and Howard, *Biochem.* 29:4175-4180 (1990)), and the intramolecular interaction between portions of the glycoprotein which can affect the conformation and presented three-dimensional surface of the glycoprotein (Jefferis and Lund, *supra*; Wyss and Wagner, *Current Opin. Biotech.* 7:409-416 (1996)). Oligosaccharides may also serve to target a given glycoprotein to certain molecules based upon specific recognition structures. For example, it has been reported that in agalactosylated IgG, the oligosaccharide moiety 'flips' out of the inter-CH2 space and terminal N-acetylglucosamine residues become available to bind mannose binding protein (Malhotra *et al., Nature Med.* 1:237-243 (1995)). Removal by glycopeptidase of the oligosaccharides from CAMPATH-1H (a recombinant humanized murine monoclonal IgG1 antibody which recognizes the CDw52 antigen of human lymphocytes) produced in Chinese Hamster Ovary (CHO) cells resulted in a complete reduction in complement mediated lysis (CMCL) (Boyd *et al., Mol. Immunol.* 32:1311-1318 (1996)), while selective removal of sialic acid residues using neuraminidase resulted in no loss of CMCL. Glycosylation of antibodies has also been reported to affect antibody-dependent cellular cytotoxicity (ADCC). In particular, CHO cells with tetracycline-regulated expression of β(1,4)-N-acetylglucosaminyltransferase III (GnTIII), a glycosyltransferase catalyzing formation of bisecting GlcNAc, was reported to have improved ADCC activity (Umana *et al., Mature Biotech.* 17:176-180 (1999)).

[0082] Glycosylation variants of antibodies can be prepared by modifying the glycosylation sites in the underlying nucleotide sequence. In addition, the glycosylation of antibodies may also be altered without altering the underlying nucleotide sequence. Glycosylation largely depends on the host cell used to express the antibody. Since the cell type used for expression of recombinant glycoproteins, e.g. antibodies, as potential therapeutics is rarely the native cell, significant variations in the glycosylation pattern of the antibodies can be expected (see, e.g. Hse *et al., J. Biol. Chem.* 272:9062-9070 (1997)). In addition to the choice of host cells, factors which affect glycosylation during recombinant production of antibodies include growth mode, media formulation, culture density, oxygenation, pH, purification schemes and the like. Various methods have been proposed to alter the glycosylation pattern achieved in a particular host organism including introducing or overexpressing certain enzymes involved in oligosaccharide production (U. S. Patent Nos. 5,047,335; 5,510,261 and 5.278,299). Glycosylation, or certain types of glycosylation, can be enzymatically removed from the glycoprotein, for example using endoglycosidase H (Endo H). In addition, the recombinant host cell can be genetically engineered, e.g. make defective in processing certain types of polysaccharides. These and similar techniques are well known in the art.

[0083] The antibodies of the present invention may also be used by the antibody-directed enzyme prodrug therapy (ADEPT). ADEPT is a technology that utilizes the specificity of monoclonal antibodies targeting tumor antigens to target catalytic enzymes to the surface of cancer cells. There, the enzymes are in position to activate prodrug forms (*e.g.*, a peptidyl chemotherapeutic agent, see WO81/01145) of anti-cancer drugs to their fully active form. See, for example, WO 88/07378 and U.S. Patent No. 4,975,278.

[0084] Enzymes that are useful in the method of this invention include, but are not limited to, alkaline phosphatase useful for converting phosphate-containing prodrugs into free drugs; arylsulfatase useful for converting sulfate-containing prodrugs into free drugs; cytosine deaminase useful for converting non-toxic 5-fluorocytosine into the anti-cancer drug, 5-fluorouracil; proteases, such as serratia protease, thermolysin, subtilisin, carboxypeptidases and cathepsins (such as cathepsins B and L), that are useful for converting peptide-containing prodrugs into free drugs; D-alanylcarboxypeptidases, useful for converting prodrugs that contain D-amino acid substituents; carbohydrate-cleaving enzymes such as β-galactosidase and neuraminidase useful for converting glycosylated prodrugs into free drugs; β-lactamase useful for converting drugs derivatized with β-lactams into free drugs; and penicillin amidases, such as penicillin V amidase or penicillin G amidase, useful for converting drugs derivatized at their amine nitrogens with phenoxyacetyl or phenylacetyl groups, respectively, into free drugs.

Alternatively, antibodies with enzymatic activity, also known in the art as "abzymes", can be used to convert the prodrugs of the invention into free active drugs (see, *e.g.*, Massey, *Nature* 328:457-458 (1987)). Antibody-abzyme conjugates can be prepared as described herein for delivery of the abzyme to a tumor cell population.

[0085] Immunoconjugates of the antibodies herein are also specifically encompassed by this invention. Immunoconjugates comprise an antibody conjugated to a cytotoxic agent, such as chemotherapeutic agent, a toxin, or a radioisotope.

[0086] Specifically, the efficacy of the anti-CD22 antibodies herein can be further enhanced by conjugation to a cytotoxic radioisotope, to allow targeting a radiotherapy specifically to target sites (radioimmunotherapy). Suitable radioisotopes include, for example, I^{131} and Y^{90}, both used in clinical practice. Other suitable radioisotopes include, without limitation, In^{111}, Cu^{67}, I^{131}, As^{211}, Bi^{212}, Bi^{213}, and Re^{186}.

[0087] Chemotherapeutic agents useful in the generation of immunoconjugates include, for example, include adriamycin, doxorubicin, epirubicin, 5-fluorouracil, cytosine arabinoside ("Ara-C"), cyclophosphamide, thiotepa, busulfan, cytoxin, taxoids, *e.g.*, paclitaxel (Taxol, Bristol-Myers Squibb Oncology, Princeton, NJ), and doxetaxel (Taxotere, Rhône-Poulenc Rorer, Antony, Rnace), toxotere, methotrexate, cisplatin, melphalan, vinblastine, bleomycin, etoposide, ifosfamide, mitomycin C, mitoxantrone, vincristine, vinorelbine, carboplatin, teniposide, daunomycin, carminomycin, aminopterin, dactinomycin, mitomycins, esperamicins (see U.S. Pat. No. 4,675,187), 5-FU, 6-thioguanine, 6-mercaptopurine, actinomycin D, VP-16, chlorambucil, melphalan, and other related nitrogen mustards.

[0088] Toxins to be used in the immunoconjugates herein include, for example, diphtheria A chain, exotoxin A chain, ricin A chain, enomycin, and tricothecenes. Specifically included are antibody-maytansinoid and antibody-calicheamicin conjugates. Immunoconjugates containing maytansinoids are disclosed, for example, in U.S. Patent Nos. 5,208,020, 5,416,020 and European Patent EP 0 425 235. See also Liu *et al., Proc. Natl. Acad. Sci. USA* 93:8618-8623 (1996). Antibody-calicheamicin conjugates are disclosed, e.g. in U. S. Patent Nos. 5,712,374; 5,714,586; 5,739,116; 5,767,285; 5,770,701; 5,770,710; 5,773,001; and 5,877,296.

[0089] Conjugates of the antibody and cytotoxic agent are made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCl), active esters (such as disuccinimidyl suberate), aldehydes (such as glutareldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6-diisocyanate),

and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta *et al.*, *Science*, 238:1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody. *See*, WO94/11026.

[0090] Covalent modifications of the anti-CD22 antibodies are also included within the scope of this invention. They may be made by chemical synthesis or by enzymatic or chemical cleavage of the antibody, if applicable. Other types of covalent modifications of the antibody are introduced into the molecule by reacting targeted amino acid residues of the antibody with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C-terminal residues. A preferred type of covalent modification of the antibodies comprises linking the antibodies to one of a variety of nonproteinaceous polymers, *e.g.*, polyethylene glycol, polypropylene glycol, or polyoxyalkylenes, in the manner well known in the art.

2. Pharmaceutical Formulations and Treatment Methods

[0091] B-cell type Non-Hodgkin's Lymphoma is a term that is used to encompass a large group (over 29 types) of lymphomas caused by malignant (cancerous) B cell lymphocytes, and represents a large subset of the known types of lymphoma. B-cells are known to undergo many changes in their life cycle dependent on complex intracellular signaling processes, and apparently different types of B-cell malignancies can occur at different stages of the life cycle of B-cells. At the stem cell stage, acute lymphocytic leukemia (ALL) or lymphoblastic lymphoma/leukemia can typically develop. Precursor B-cells can develop precursor B lymphoblastic lymphoma/leukemia. Typical malignancies of immature B-cells include small non-cleaved cell lymphoma and possibly Burkitt's/non-Burkitt's lymphoma. B cells before antigen exposure typically develop chronic lymphocytic leukemia (CLL) or small lymphocytic lymphoma, while after antigen exposure typically follicular lymphomas, large cell lymphoma and immunoblastic lymphoma are observed. There are also classification systems that characterize B-cell lymphomas by the rate of growth distinguishing aggressive (fast growing) and indolent (slow growing) lymphomas. For example, Burkitt's/non-Burkitt's lymphoma and LCL lymphoma belong in the aggressive group, while indolent lymphomas include follicular center cell lymphomas (FCCL), follicular large cell lymphomas, and follicular small cleaved cell lymphomas.

[0092] Non-Hodgkin's Lymphomas are also characterized by the stage of development. Stage I: cancer is found in only one lymph node area, or in only one area or organ outside the lymph nodes. Stage II: (1) Cancer is found in two or more lymph node areas on the

same side of the diaphragm (the thin muscle under the lungs that helps breathing), or, (2) cancer is found in only one area or organ outside the lymph nodes and in the lymph nodes around it, or (3) other lymph node areas on the same side of the diaphragm may also have cancer. Stage III: Cancer is found in lymph node areas on both sides of the diaphragm. The cancer may also have spread to an area or organ near the lymph node areas and/or to the spleen. Stage IV: (1) Cancer has spread to more than one organ or organs outside the lymph system; cancer cells may or may not be found in the lymph nodes near these organs, or (2) cancer has spread to only one organ outside the lymph system, but lymph nodes far away from that organ are involved.

[0093] Current treatment options of B-cell malignancies, including non-Hodgkin's lymphomas depend on the type and stage of malignancy. Typical treatment regimens include radiation therapy, also referred to as external beam therapy, chemotherapy, immunotherapy, and combinations of these approaches. One promising approach is radioimmunotherapy (RIT). With external beam therapy, a limited area of the body is irradiated. With chemotherapy, the treatment is systemic, and often adversely affects normal cells, causing severe toxic side-effects. Targeted RIT is an approach in which a B-cell specific antibody delivers a toxic substance to the site of tumor. The therapeutic potential of RIT in patients with B-cell NHL has been shown using different targets, including CD20, CD19, CD22, and HLA-DR10 (Lym-1). More recently, combined modality therapy (CMT) has become an increasingly frequent maneuver for the treatment of solid tumors, and includes radiosensitization of cancer cells by drugs, and the direct cytotoxic effect of chemotherapy. The most common chemotherapy regiment for treating NHL is Cyclophosphamide-Hydroxydoxorubicin-Oncovin (vincristine)-Prednisone (CHOP) combination therapy. A randomized study of aggressive, but early stage NHL showed superior results with CHOP plus involved field radiation over treatment with CHOP alone. Despite its promise, the disadvantage of treatments involving external beam radiation is that external beam radiation can only be delivered in high doses to a limited region of the body, while NHL is mostly widespread. Accordingly, CMT has proven clinically useful for locally advanced malignancies.

[0094] Another current approach is combined modality radioimmunotherapy (CMRIT), which pairs the specific delivery of systemic radiation (e.g. ^{90}Y-DOTA-peptide-Lym-1) to NHL with the systemic radiation sensitizing effects of an additional chemotherapeutic agent. Because in CMRIT radiation is delivered continuously, cancer cells that are hypoxic may re-oxygenate, or pass through the radiosensitive G_2/M phase of the cell cycle during the course of treatment, making cure more likely. In addition, CMRIT provides specificity first, by the specific targeting of NHL by Lym-1, and second by timing. This allows the radiation sensitizer to potentially synergize only at the sites targeted by RIT, thus maximizing efficacy and minimizing

toxicity. Several previous xenograft studies have demonstrated improved synergy when the radiation synthesizer (Taxol) was given 24-48 hours after RIT.

[0095] Although CMRIT is currently viewed as the most advanced therapeutic approach for the treatment of NHL, the antibodies of the present invention alone have been demonstrated to provide superior results both in terms of tumor volume reduction, cure rate and overall survival, when tested in the well accepted Raji and Ramos lymphoma xenograft models.

[0096] The anti-CD22 antibodies herein are typically administered in the form of pharmaceutical formulations well known to all pharmaceutical chemists. See, e.g. Remington's Pharmaceutical Sciences, (15th Edition, Mack Publishing Company, Easton, Pa. (1975)), particularly Chapter 87, by Blaug, Seymour. These formulations include for example, powders, pastes, ointments, jelly, waxes, oils, lipids, anhydrous absorption bases, oil-in-water or water-in-oil emulsions, emulsions carbowax (polyethylene glycols of a variety of molecular weights), semi-solid gels, and semi-solid mixtures containing carbowax. A typical dosage form is a sterile, isotonic, water-based solution suitable for administration by the intravenous (i.v.) route. The concentration of the antibodies of the invention in the pharmaceutical formulations can vary widely, i.e., from less than about 0.1%, usually at or at least about 2% to as much as 20% to 50% or more by weight, and will be selected primarily by fluid volumes, viscosities, etc., in accordance with the particular mode of administration selected.

[0097] The compositions of the invention may also be administered via liposomes. Liposomes include emulsions, foams, micelles, insoluble monolayers, liquid crystals, phospholipid dispersions, lamellar layers and the like. In these preparations the composition of the invention to be delivered is incorporated as part of a liposome, alone or in conjunction with a molecule which binds to a desired target, such as antibody, or with other therapeutic or immunogenic compositions. Liposomes for use in the invention are formed from standard vesicle-forming lipids, which generally include neutral and negatively charged phospholipids and a sterol, such as cholesterol. The selection of lipids is generally guided by consideration of, e.g., liposome size, acid lability and stability of the liposomes in the blood stream. A variety of methods are available for preparing liposomes, as described in, e.g., Szoka et al. *Ann. Rev. Biophys. Bioeng.* 9:467 (1980), U.S. Pat. Nos. 4,235,871, 4,501,728, 4,837,028, and 5,019,369.

[0098] The antibodies of the present invention can be administered alone or in combination with other therapeutic regimens, including chemotherapy, radioimmunotherapy (RIT), chemotherapy and external beam radiation (combined modality therapy, CMT), combined modality radioimmunotherapy (CMRIT), or cytokines alone or in combination, etc. Thus, the anti-CD22 antibodies of the present invention can be combined with CHOP (Cyclophosphamide-

Hydroxydoxorubicin-Oncovin (vincristine)-Prednisolone), the most common chemotherapy regimen for treating non-Hodgkin's lymphoma. In addition, the anti-CD22 antibodies herein may be administered in combination with other antibodies, including anti-CD19, anti-CD20 and other anti-CD22 antibodies, such as LymphoCide™ (Immunomedics, Inc.) or LymphoCide Y-90. See, for example, Stein *et al., Drugs of the Future* 18:997-1004 (1993); Behr *et al., Clinical Cancer Research* 5:3304s-33314s, 1999 (suppl.); Juweid *et al., Cancer Res.* 55:5899s-5907s, 1995; Behr *et al., Tumor Targeting* 3:32-40 (1998), and U.S. Pat. Nos. 6,183,744, 6,187,287, and 6,254,868.

[0099] The patients to be treated in accordance with the present invention will have CD22 expressed on their malignant B cells. The presence of the CD22 antigen can be confirmed by standard techniques, such as immunohistochemistry, FACS, binding assay with labeled (e.g. radiolabeled) anti-CD22 antibody.

[0100] The preferred route of administration is via bolus or continuous infusion over a period of time, such as continuous or bolus infusion, once or twice a week. Another preferred route is subcutaneous injection. The dosage depends on the nature, form, and stage of the targeted B cell malignancy, the patients sex, age, condition, prior treatment history, other anti-cancer treatments used (including, e.g. radiation, chemotherapy, immunotherapy, etc.) and other factors typically considered by a skilled physician. For example, non-Hodgkin's lymphoma patients may receive from about 50 to about 1500 mg/m^2/week, specifically from about 100 to about 1000 mg/m^2/week, more specifically from about 150 to about 500 mg/m^2/week of an anti-CD22 antibody herein.

[0101] The patients will be monitored by standard techniques, such as by monitoring tumor regression, e.g. tumor size in the case of solid tumors, the phenotype of circulating B-cells or of biopsied tissues using anti-CD22 antibodies.

[0102] While the invention has been discussed with reference to human therapy, it will be understood that the antibodies of the present invention also find use in veterinary medicine. For example, feline malignant lymphoma occurs frequently in domestic cats, and shows similar characteristics to human non-Hodgkin's lymphoma (Bertone *et al., Am. J. Epidemiol.*156:268-73 (2002)). Similarly, dogs are known to develop a variety of lymphomas. Accordingly, the antibodies herein can be used to treat feline and canine malignant lymphoma. Dosages, and routes of administration depend on the animal species to be treated, and their determination is well within the skill of a veterinary of ordinary skill.

[0103] Further details of the invention are provided in the following non-limiting examples.

EXAMPLES

[0104] Commercially available reagents referred to in the examples were used according to manufacturer's instructions unless otherwise indicated. In addition to production as disclosed in the following examples, hybridoma producing monoclonal antibody HB22-7 (ATCC Accession No. HB11349) may be obtained from the American Type Culture Collection, Rockville, MD.

EXAMPLE 1

Production of anti-CD22 monoclonal antibodies

[0105] Monoclonal antibodies (mAbs) HB22-7 (IgG2b), HB22-23 (IgG2a) HB22-33 (IgM), HB22-5 (IgG2a), HB22-13 (IgG2a), HB22-22 (IgA), and HB22-196 were produced according to the method of Engel *et al., J Immunol* 15:4710 (1993) and U.S. Pat. No. 5,484,892. See, also Tuscano *et al., Blood* 94:1382-1392 (1999). However, other methods may be used. Briefly, the HB22 mAbs were produced via hybridoma techniques using a mouse pre-B cell line 300.19, stably transfected with full length CD22 cDNA, as the immunogen. More specifically, thirty-three mAbs reactive with CD22 were generated by the fusion of NS-1 myeloma cell with spleen cells from Balb/c mice immunized three times with a mouse pre-B cell line, 300.19, stably transfected with a full-length CD22 cDNA. Hybridomas producing mAb reactive with mouse L cells transfected with CD22 cDNA, but not with untransfected cells, were cloned twice and used to generate supernatant or ascites fluid. mAb isotypes were determined using the Mouse Monoclonal Antibody Isotyping Kit (Amersham, Arlington Heights, Ill.). IgGmAb were purified using the Affi-Gel Protein A MAPS II Kit (Bio-Rad, Richmond, Calif.). The HB22-33 mAb (IgM) containing euglobulin fraction of ascites fluid was precipitated by extensive dialysis against distilled water and was shown to be essentially pure mAb by SDS-PAGE analysis. As disclosed in Table II of U.S. Pat. No. 5,484,892, mAbs HB22-7, HB22-22, HB22-23, and HB22-33 completely blocked (80-100%) the binding of Daudi, Raji and Jurkat cells to CD22 transfected COS cells. mAbs HB22-5, HB22-13, HB22-24, and HB22-28 partially blocked adhesion (20-80%).

[0106] The region(s) on CD22 that mediates ligand binding was characterized by mAb cross-inhibition studies using the "Workshop" CD22-blocking mAb and a panel of mAb that identify five different epitopes on CD22 (epitopes A, B, C, D, and E (Schwartz-Albiez *et al.*, "The carbohydrate moiety of the CD22 antigen can be modulated by inhibitors of the glycosylation pathway." The binding specificities of the Workshop mAb are depicted pictorially in Fig. 3. In Leukocyte Typing IV. White Cell Differentiation Antigens, Knapp *et al., eds.*, Oxford University

Press, Oxford, p. 65 (1989)). It has been found that three of the monoclonal antibodies herein, HB22-7, HB22-22, and HB22-23, bind to very close or the same epitopes on CD22. Results of the epitope-mapping of these and other blocking antibodies are disclosed in Tedder *et al., Annu. Rev. Immunol.* 15:481-504 (1997). Unlike other anti-CD22 antibodies proposed for therapy, the blocking antibodies of the present invention bind to an epitope within the first two Ig-like domains of the hCD22 amino acid sequence.

EXAMPLE 2

Raji and Ramos Lymphoma Xenograft Trials

[0107] This example describes the results from our independent Raji and Ramos lymphoma xenograft trials. Nude mice xenografts are important tools for preclinical evaluations. Nude mice bearing human non-Hodgkin's lymphoma (NHL) xenografts utilizing the lymphoma cell lines Raji and Ramos have proven utility for evaluating efficacy for treatment of NHL . (Buchsbaum *et al., Cancer Res.* 52(23):6476-6481 (1992) and Flavell *et al., Cancer Res.* 57:4824-4829 (1997)).

Materials and Methods

[0108] *Reagents.* Carrier-free ^{90}Y (Pacific Northwest National Laboratory, Richland, WA) and ^{111}In (Nordion, Kanata, Ontario, Canada) were purchased as chlorides in dilute HCl. Lym-1 (Techniclone, Inc Tustin, CA) is an IgG_{2a} mAb generated in mice immunized with human Burkitt's lymphoma cell nuclei. Lym-1 recognizes a cell surface 31-35 kD antigen on malignant B cells, and reacts with greater than 80% of human B cell NHL. Lym-1 purity was assessed according to the specifications that required greater than 95% pure monomeric IgG by polyacrylamide gel electrophoresis. ^{90}Y-DOTA-peptide-Lym-1 was prepared as previously described (O'Donnell *et al., Cancer. Biother. Radiopharm.* 13:251-361 (1998)). Assessment by HPLC, TLC, and cellulose acetate electrophore.sis revealed that ^{90}Y-DOTA-peptide-Lym-1 was prepared to 98% radiochemical purity with less than 5% aggregate content.

[0109] The anti-CD22 mAb, HB22-7, was prepared as previously described (Tuscano *et al., Blood* 94:1382-1392 (1999)), using a Protein A Sepharose Fast Flow column (Pharmacia). HB22-7 purity was determined by HPLC and flow cytometry, and found to be >95% pure. Physiologic properties were determined by flow cytometric-based analysis of apoptotic induction (Apo-Tag, Pharmacia) and found to be consistent with previous published results (Tuscano *et al., supra*). Endotoxin removal was achieved using an ActiClean ETOX column (Sterogene), with final endotoxin levels determined to be < 0.15 Endotoxin Units (EU)/mg mAb (Bio Whitaker). The Lym-1 and HB22-7 mAbs met MAP (mouse antibody production) guidelines for murine,

viral, mycoplasma, fungal, and bacterial contamination, as well as endotoxin, pyrogen and DNA content and general safety testing in animals.

[0110] *Cell lines and Scatchard Analysis.* Raji and Ramos Burkitt lymphoma cell lines were purchased from American Type Culture Collection (ATCC, Gathersberg, MD). Both cell lines stained for CD22 expression by flow cytometric methods utilizing the HB22-7 mAb, as described previously (Tuscano *et al., supra*). The cell lines were maintained in RPMI 1640 supplemented with 10% fetal calf serum at 0.5 $x10^6$ cells/ml. A Scatchard analysis using Raji and Ramos cells was performed as described previously (Scatchard, G., *Ann. of NY Acad Sci.* 51:660 (1947)). Briefly, HB22-7 was labeled with ^{125}I by the chloramine T method (specific activity of 1.1μCi/μg). A competitive binding assay was performed utilizing serially diluted, unlabeled HB22-7.

[0111] *Mouse studies.* Female athymic BALB/c nu/nu mice (Harlan Sprague-Dawley), 7-9 weeks of age were maintained according to University of California, Davis animal care guidelines on a normal diet ad libitum and under pathogen-free conditions. Five mice were housed per cage. Raji or Ramos cells were harvested in logarithmic growth phase; 2.5-5.0 X 10^6 cells were injected subcutaneously into both sides of the abdomen of each mouse. Studies were initiated 3 weeks after implantation, when tumors were 28-328 mm^3. Groups consisted of untreated, 125μCi of RIT alone, 1.4 mg of HB22-7 alone, or the combination of RIT and HB22-7, with HB22-7 being administered 24 hours prior, simultaneously, or 24 hours after RIT. To minimize ambient radiation, bedding was changed daily for 1 week after treatment with ^{90}Y-DOTA-peptide-Lym-1, and twice weekly thereafter.

[0112] *Tumoricidal Effect.* Tumor volume was calculated as described by the formula for hemiellipsoids (DeNardo *et al., Clin. Cancer Res.* 3:71-79 (1997)). Initial tumor volume was defined as the volume on the day prior to treatment. Mean tumor volume was calculated for each group on each day of measurement; tumors that had completely regressed were considered to have a volume of zero. Tumor responses were categorized as follows: C, cure (tumor disappeared and did not regrow by the end of the 84 day study); CR, complete regression (tumor disappeared for at least 7 days, but later regrew); PR, partial regression (tumor volume decreased by 50% or more for at least 7 days, then regrew).

[0113] *Statistical Analysis.* Differences in response among treatment groups were evaluated using the Kruskall Walis rank sum test with the response ordered as none, PR, CR, and Cure. Survival time was also evaluated using the Kruskall Walis test. Tumor volume was compared at 3 time points: month 1 (day 26-29), month 2 (day 54-57), and at the end of the study (day 84). If an animal was sacrificed due to tumor-related causes, the last volume was carried

forward and used in the analysis of later time points. Analysis of variance was used to test for differences among treatment groups. P values are two-tailed and represent the nominal p-values. Protection for multiple comparisons is provided by testing only within subsets of groups found to be statistically significantly different.

Results

Scatchard Analysis

[0114] Scatchard analysis was utilized to assess the binding affinity of HB22-7 and the number of CD22 receptors on Ramos and Raji cells. The cells were assayed for maximum binding percentage (Bmax), disassociation constant (Ka) and number of antibodies bound per cell. The results shown in Table 1 are the average of two experiments.

Table 1

I. PARAMETER	Cell Lines	
Cell line	Raji	Ramos
Bmax	53.5 ±0.9%	21.0 ± 1.3%
R^2	0.954	0.926
Ka	1.3 ±0.08 X 10^9	5.95 ±1.0 X 10^8
Antibody/cell	118,000	43,000

[0115] The Scatchard analysis (Table 1) revealed a nearly 2.5 fold increase in the number of HB22-7 antibodies bound per cell, and Bmax, and a 2 fold increase in Ka for Raji cells versus Ramos cells, respectively.

Whole Body Autoradiography

[0116] In order to assess HB22-7-specific tumor targeting, whole body autoradiography of tumor-bearing nude mice injected with ^{111}In-2IT-BAD-anti-CD22 (HB22-7) was performed. Forty eight hours after injection mice were sacrificed, sectioned and autoradiographed (Figure 2), as previously described (DeNardo *et al., Cancer* 3:71-79 (1997)). Autoradiography revealed intense tumor localization in the Raji-tumored mice and moderate localization in the Ramos-tumored mice. This targeting study is consistent with the Scatchard analysis that revealed less HB22-7 bound per Ramos cells as compared to Raji. However the rapid growth of Ramos tumors, and likely central necrosis, may also contribute to the apparent inferior targeting of Ramos.

Efficacy of RIT and CMRIT

[0117] The initial trial (081500) utilized 125 uCi of ^{90}Y-DOTA-peptide-Lym-1 alone or in combination with HB22-7 (1.4 mg) given either 24 hours prior, simultaneously, or 24 hours after RIT, (Figure 3). In this trial there were 5 mice per group with the exception of the group treated with RIT alone, which had 9 mice and 5 untreated controls (mouse numbers are tabulated in Table 2).

Table 2

Trial	Treatment Groups					
	No Tx	HB22-7	RIT	-24	@RIT	+24
081500	5	4	9	5	5	5
101600	5	6	5	5	3	5
011601	_	5	4	_	9	7
032701	_	5	2	_	3	12
052401	3	_	3	_	_	_
060401	5	5	_	_	_	_
071701	7	5	_	_	_	4
092101	4	_	_	_	_	_
102401	13	_	_	_	_	_
Total	42	30	23	10	20	33

As predicted from similar Raji xenograft studies with ^{90}Y-2IT-BAD-Lym-1, RIT alone resulted in maximal mean tumor volume reduction by day 21, with increasing tumor volume thereafter. Xenografts treated with ^{90}Y-2IT-BAD-Lym-1(RIT) and HB22-7 (CMRIT) demonstrated greater and more sustained mean tumor volume reduction, which was greatest when HB22-7 was administered simultaneously, and 24 hours after RIT. Surprisingly, HB22-7 administered alone resulted in stabilization of mean tumor volume by 2-3 weeks, then a gradual and sustained tumor volume reduction.

[0118] Several additional replicate trials were conducted with highly reproducible results (Table 2). The data from all trials were compiled and, when compared graphically, revealed results highly consistent with the initial study, (Figure 4). The initial tumor volume reductions were again greatest at approximately day 21 when HB22-7 was administered simultaneously and 24 hours after RIT. In mice treated with HB22-7 alone, the stabilization in

tumor growth that began 2 weeks after treatment followed by gradual sustained tumor volume reduction was also replicated in all subsequent trials. Using analysis of variance, when examining all treatment groups at day 30 the differences were highly significant ($p<0.001$). While analysis of volume reduction in all treatment groups at day 60 did not demonstrate significant differences ($p=0.39$), the differences at day 84 again were significant ($p=0.003$). The results observed graphically revealed that the difference in volume reduction in the RIT/CMRIT groups was highly reproducible and different from HB22-7 alone and untreated control, however, comparison of volume reduction only in only RIT treatment groups (including CMRIT) at all time points assessed (day 30, 60, and 84) did not reveal significant differences ($p\geq 0.5$). Additional CMRIT trials were done with HB22-7 being administered 48 and 72 hours after RIT. The extended interval between the administration of RIT and HB22-7 did not result in improved tumor volume reduction when compared to trials in which HB22-7 was given simultaneously and 24 hours after RIT (data not shown).

[0119] Response and cure rates were consistent with the effects of treatment on tumor volume, (Figure 5). Treatment with ^{90}Y-DOTA-peptide-Lym-1 alone produced 48% PR, 13% CR, and a 13% cure rate. In the CMRIT groups, the overall response rate was maximized when HB22-7 and RIT were administered simultaneously generating 45% PR, 15% CR and 25% cure. However in the CMRIT groups the cure rate was the greatest (39%) when HB22-7 was administered 24 hours after RIT, which compared favorably to the cure rates observed in the untreated (29%), RIT alone (13%), 24 hours prior (10%) and simultaneous (25%) treatment groups. When examining the degree of response (ranking cure better than CR, better than PR) in all treatment groups using the Kruskal Walis test, the differences were statistically significant ($p=0.01$). Individual comparisons against untreated controls were all statistically significant ($p<0.05$), with the exception of RIT alone ($p=0.06$) and HB22-7 given 24 hours prior to RIT ($p=0.16$). While comparison of only active treatment groups (RIT alone, CMRIT, and HB22-7) was not significantly different ($p=0.18$), the CMRIT groups treated with HB22-7 simultaneously and after 24 hours had the best observed pattern of response. Interestingly the group treated with HB22-7 alone had the highest cure rate (47%) which was a significant improvement when compared to the untreated controls ($p<0.05$).

[0120] Tumor volume regression and cure rates translated into a similar pattern of survival. At the end of the 84 day study period 38 and 42% of the untreated and RIT alone groups were alive respectively, (Figure 6). In the CMRIT treatment groups, survival increased to 67 and 50% when HB22-7 was administered simultaneously and 24 hours after RIT, respectively. Analysis of survival using Kruskal Walis was significant ($p< 0.05$) for comparison of all groups.

Similar to the response rate analysis, comparison of survival in the RIT groups only did not reveal significant differences (p=0.41), however the best survival in these groups was consistently observed when HB22-7 was administered either simultaneous or 24 hours after RIT.

[0121] The best overall survival, 76%, was observed in the group treated with HB22-7 alone, a significant difference when compared to untreated control (p=0.02).

Toxicity

[0122] Hematologic and non-hematologic toxicities were assessed by blood counts and mouse weights, respectively (Figure 7a-c). WBC and platelet nadirs in the RIT treatment groups were at 14-20, and 10-14 days respectively. WBC and platelet recovery was approximately 28 and 21 days after treatment, respectively. The WBC and platelet nadirs were consistent with observations in previous studies that utilized 150uCi of ^{90}Y-2IT-BAD-Lym-1. The hematologic toxicity of RIT was not altered by co-administration of HB22-7. No hematologic toxicity was detected in mice treated with HB22-7 alone. Analysis of mononuclear cell counts in all treatment groups revealed that HB22-7 had no effect on RIT-mediated mononuclear cell nadirs (data not shown). Non-hematologic toxicity as assessed by changes in mouse weight, and was found to be equivalent in all treatment groups (Figure 8). There were no deaths due to toxicity in any treatment groups.

^{90}Y-DOTA-peptide-Lym-1 Pharmacokinetics

[0123] Blood and whole body clearances of ^{90}Y-DOTA-peptide-Lym-1 in Raji-tumored mice with or without HB22-7 were similar (Figure 9). The blood biological $T_{1/2}\,\alpha$ was 1.4 hours for RIT alone, and 2.2, 2.4, and 2.0 hours for the 24 hour prior, simultaneous and 24 hour after groups respectively. The blood biological $T_{1/2}\,\beta$ was 127 hours for the RIT alone group and 133, 87, and 103 hours for the 24 hours prior, simultaneous and 24 hours after groups respectively. The whole body $T_{1/2}$ was 246 hours for RIT alone and 207, 207, and 196 hours for the 24 hours prior, simultaneous and 24 hours after groups respectively. The addition of HB22-7 to RIT did not change the pharmacokinetics of ^{90}Y-DOTA-peptide-Lym-1.

Discussion

[0124] Raji xenograft studies were designed to determine if the anti-CD22 mAb (HB22-7) would generate additive or synergistic effects when combined with RIT to enhance apoptosis and/or DNA damage induced by low dose-rate radiation. The Raji xenograft nude mouse model has proven useful when used to assess toxicity and efficacy of RIT using ^{90}Y-2IT-

BAD-Lym-1 RIT alone (O'Donnell *et al., Cancer Biotherapy and Radiopharmaceuticals* 13:351-361 (1998)). Responses in this pre-clinical model translated into significant efficacy in human clinical trials (O'Donnell *et al., Anticancer Res.* 20:3647-55 (2000); O'Donnell *et al., J. Nucl. Med.* 40:216 (1999) (Abstract)).

[0125] In the studies described in this Example, the addition of the anti-CD22 mAb HB22-7 to ^{90}Y-DOTA-peptide-Lym-1(125uCi) enhanced the efficacy of RIT without any change in toxicity. Previous Raji xenograft studies with 150 and 200μCi of ^{90}Y-2IT-BAD-Lym-1 generated response and cure rates that were comparable to those observed in the present study (O'Donnel *et al.,* (1998), *supra*). The 125 μCi dose of ^{90}Y-DOTA-peptide-Lym-1 was chosen based on these previous studies with the 2IT-BAD linker. While the previous studies with 2IT-BAD demonstrated greatest efficacy with the 200 μCi dose, the choice of 125 μCi was based on the hypothesis that HB22-7 would be synergistic or additive with RIT and the lower dose would allow for better assessment of these effects. The studies of this Example utilized a novel linker (DOTA-peptide) that has not been previously examined in lymphoma xenograft models. The DOTA-peptide linker was designed for enhanced hepatic degradation of unbound radiopharmaceutical thereby leading to a more favorable biodistribution. While tumor-specific uptake was not assessed in detail in this study, the toxicity profile observed with 125 uCi of ^{90}Y-DOTA-peptide-Lym-1 alone was acceptable with no treatment-related mortality and predictable leukocyte and platelet nadirs.

[0126] HB22-7 was chosen based on *in vitro* studies demonstrating pro-apoptotic and signaling effects (Tuscano *et al., Blood* 94:1382-1392 (1999)). The treatment dose of HB22-7 utilized was empiric, however, it was based on the amount that was shown to be effective at inducing apoptosis *in vitro* and extrapolating this to the mouse model. In addition, when formulating the dose of HB22-7 consideration was given to the equivalent (when adjusted for body surface area differences in humans versus mice) dose of Rituximab® used in human clinical trials. The approximation to the Rituximab® dose was utilized based on the fact that this is the only naked mAb available that has demonstrated efficacy for the treatment of lymphoma, granted, the optimal dose of Rituximab® is currently undefined.

[0127] The study was designed to assess the efficacy of HB22-7 alone, the combination of RIT and HB22-7 as well as the effect of three different sequence combinations. The tumor volume reduction observed with ^{90}Y-DOTA-peptide-Lym-1 alone was consistent with previous studies with ^{90}Y-2IT-BAD-Lym-1 in terms of timing, magnitude, and duration of response (O'Donnel *et al.,* 1998, *supra*). RIT alone resulted in approximately 50% reduction in tumor volume 14 days after therapy. When assessing at the approximate point of maximal volume

reduction (day 21-30) the addition of HB22-7 to RIT significantly enhanced the magnitude of response in a sequence specific manner. It appears that the addition of HB22-7 was most effective when administered simultaneously or 24 hours after RIT. The distinctive pattern of volume reduction was highly reproducible. Independent replicate trials demonstrated similar patterns and magnitude of tumor volume reduction. The improved reductions in tumor volume translated into superior response rates and survival. RIT alone generated 13% CR and 13% cures, the addition of HB22-7 increased the cure rate to 25% when administered simultaneously with RIT, and to 39% when HB22-7 was administered 24 hours after RIT.

[0128] This is the first time that a second monoclonal antibody has been combined with RIT, and demonstrates the potential of utilizing monoclonal antibodies or other agents with well defined physiologic properties that may augment efficacy without increasing toxicity.

[0129] Surprisingly the mice treated with HB22-7 alone had impressive tumor volume reduction and superior cure and survival rates when compared to all other treatment groups. Again, several independent trials generated highly consistent results with a delayed initial tumor volume stabilization, and then tumor volume reduction beginning approximately 14 days after treatment. This translated into the best cure and overall survival rates observed in any of the treatment groups.

[0130] In conclusion, the antibodies of the present invention, when administered alone, have been demonstrated to provide superior results in terms of tumor volume reduction, cure rate and overall survival when compared to other treatment regimens, including CMRIT, which is currently viewed as the most advanced therapeutic approach for the treatment of NHL.

EXAMPLE 3

Sequence Analysis of anti-CD22 Antibodies

V_H and Light Chain Gene Utilization

[0131] Cytoplasmic RNA was extracted from 1-10 x 10^5 hybridoma cells using the RNeasy Mini Kit (Qiagen Chatsworth, CA). First strand cDNA was synthesized from cytoplasmic RNA using oligo-dT primers (dT_{18}) and a Superscript Kit (Gibco BRL, Gaithersburg, MD). One µl of cDNA solution was used as template for PCR amplification of V_H genes. PCR reactions were carried out in a 100-µl volume of a reaction mixture composed of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM dNTP (Perkin Elmer, Foster City, CA), 50 pmol of each primer, and 5 U of Taq polymerase (ISC Bioexpress, Kaysville, UT). Amplification was for 30 cycles (94°C for 1 min, 58° for 1 min, 72°C for 1 min; Thermocycler, Perkin Elmer). *V_H* genes were amplified using a promiscuous sense 5' V_H primer (Ms V_HE: 5' GGG AAT TCG

AGG TGC AGC TGC AGG AGT CTG G 3'; SEQ ID NO: 2) as previously described (Kantor *et al., J. Immunol.* 158:1175-86 (1996)), and antisense primers complementary to the Cμ coding region (primer Cμ-in: 5' GAG GGG GAC ATT TGG GAA GGA CTG 3'; SEQ ID NO: 3) or the Cγ region (Primer Cγ1: 5' GAG TTC CAG GTC ACT GTC ACT GGC 3'; SEQ ID NO: 4).

[0132] Light chain cDNA was amplified using a sense Vκ primer [5' ATG GGC (AT)TC AAG ATG GAG TCA CA(GT) (AT)(CT)(CT) C(AT)G G 3'; SEQ ID NO: 5] and a Cλ antisense primer (5' ACT GGA TGG TGG GAA GAT G 3'; SEQ ID NO: 6).

[0133] HB22-33 light chain sequences were amplified using a different sense Vκ primer (5' ATG AAG TTG CCT GTT AGG CTG TTG GTG CTG 3'; SEQ ID NO: 7).

[0134] Amplified PCR products were purified from agarose gels using the QIAquick gel purification kit (Qiagen) and were sequenced directly in both directions using an ABI 377 PRISM DNA sequencer after amplification using the Perkin Elmer Dye Terminator Sequencing system with AmpliTaq DNA polymerase and the same primers for initial PCR amplification. All V_H and light chain regions were sequenced completely on both the sense and anti-sense DNA strands.

[0135] The alignment of the V_H and Vκ amino amino acid sequences for anti-CD22 monoclonal antibodies HB22-5, HB22-7, HB22-13, HB22-23, HB22-33, and HB22-196 are shown in Figures 10 and 17, respectively. Figures 11-16 show the nucleotide and amino acid sequences for heavy chain V_H-D-J_H junctions of anti-CD22 Abs from hybridomas HB22-5 (SEQ ID NOS: 8 and 9), HB22-7 (SEQ ID NOS: 10 and 11); HB-22-13 (SEQ ID NOS: 12 and 13); HB-22-23 (SEQ ID NOS: 14 and 15); HB-22-33 (SEQ ID NOS: 16 and 17); and HB-22-196 (SEQ ID NOS: 18 and 19). Figures 18-23 show the nucleotide and deduced amino acid sequences for kappa light chain V-J-constant region junctions of anti-CD22 Abs from hybridomas HB22-5 (SEQ ID NOS: 20 and 21); HB22-7 (SEQ ID NOS: 22 and 23); HB22-13 (SEQ ID NOS: 24 and 25) HB22-23 (SEQ ID NOS: 26 and 27); HB22-33 (SEQ ID NOS: 28 and 29); and HB22-196 (SEQ ID NOS: 30 and 31).

WHAT IS CLAIMED IS:

1. A method for treating a human patient diagnosed with a B-cell malignancy, comprising (1) administering to said human patient an effective amount of a blocking anti-CD22 monoclonal antibody binding to the first two Ig-like domains, or to an epitope within the first two Ig-like domains of native human CD22 (hCD22) of SEQ ID NO: 1, and (2) monitoring the response of said malignancy to said treatment.

2. The method of claim 1 wherein said antibody binds to essentially the same epitope of an antibody selected from the group consisting of HB22-7 (HB11347), HB22-23 (HB11349), HB22-33, HB22-5, HB22-13, and HB22-196.

3. The method of claim 2 wherein said antibody binds to essentially the same epitope as an antibody selected from the group consisting of HB22-7 (HB11347), HB22-23 (HB11349), and HB22-33.

4. The method of claim 3 wherein said antibody binds to essentially the same epitope as HB22-7.

5. The method of claim 3 wherein said antibody binds to essentially the same epitope as HB22-33.

6. The method of claim 1 wherein said antibody blocks CD22 binding to its ligand by at least about 70%.

7. The method of claim 1 wherein said antibody blocks CD22 binding to its ligand by at least about 80%.

8. The method of claim 1 wherein said B-cell malignancy is localized.

9. The method of claim 1 wherein said B-cell malignancy is selected from the group consisting of B-cell subtype of non-Hodgkin's lymphoma, Burkitt's lymphoma, multiple myeloma, chronic lymphocytic leukemia, hairy cell leukemia, and prolymphocytic leukemia.

10. The method of claim 1 wherein said treatment is unaccompanied by any other treatment of malignant B cells.

11. The method of claim 1 wherein said treatment is unaccompanied by radiation therapy.

12. The method of claim 1 wherein said treatment is unaccompanied by chemotherapy.

13. The method of claim 1 wherein said treatment is unaccompanied by radioimmunotherapy (RIT) or combined modality radioimmunotherapy (CMRIT).

14. The method of claim 10 wherein treatment with said antibody alone provides improved cure rate in a Raji lymphoma xenograft model when compared to combination treatment with said antibody and radioimmunotherapy.

15. The method of claim 10 wherein treatment with said antibody alone provides increased survival in a Raji lymphoma xenograft model when compared to combination treatment with said antibody and radioimmunotherapy.

16. The method of claim 10 wherein treatment with said antibody alone provides superior tumor volume reduction in a Raji lymphoma xenograft model when compared to combination treatment with said antibody and radioimmunotherapy.

17. The method of claim 1 wherein said antibody is a fragment of a complete antibody.

18. The method of claim 17 wherein said antibody is selected from the group consisting of Fab, Fab', $F(ab')_2$, and Fv fragments, diabodies, linear antibodies, single-chain antibody molecules, and multispecific antibodies formed from antibody fragments.

19. The method of claim 1 wherein said antibody has an additional antigen-specificity.

20. The method of claim 19 wherein said antibody is a bispecific antibody.

21. The method of claim 20 wherein said antibody additionally binds to another epitope of CD22.

22. The method of claim 1 wherein said antibody is chimeric.

23. The method of claim 1 wherein said antibody is humanized.

24. The method of claim 1 wherein said antibody is human.

25. The method of claim 1 wherein said antibody is administered intravenously.

26. The method of claim 25 wherein said antibody is administered by weekly intravenous infusions.

27. The method of claim 6 wherein the response to said treatment is monitored by following shrinkage of a solid B-cell tumor.

28. The method of claim 27 wherein shrinkage is monitored by magnetic resonance imaging (MRI).

29. The method of claim 1 wherein said antibody comprises a heavy chain comprising a V_H sequence having at least about 95 % sequence identity with the sequence of amino acids 1 to 100 of SEQ ID NO: 9 (HB22-5 V_H sequence); or amino acids 1 to 97 of SEQ ID NO: 11 (HB22-7 V_H sequence); or amino acids 1 to 100 of SEQ ID NO: 13 (HB22-3 V_H sequence); or amino acids 1 to 100 of SEQ ID NO: 15 (HB22-23 V_H sequence); or amino acids 1 to 98 of SEQ ID NO: 17 (HB22-33 V_H sequence); or amino acids 1 to 100 of SEQ ID NO: 19 (HB22-196 V_H sequence).

30. The method of claim 29 wherein said antibody comprises a heavy chain comprising a V_H sequence having at least about 95 % sequence identity with the sequence of amino acids 1 to 97 of SEQ ID NO: 11 (HB22-7 V_H sequence); or amino acids 1 to 100 of SEQ ID NO: 15 (HB22-23 V_H sequence); or amino acids 1 to 98 of SEQ ID NO: 17 (HB22-33 V_H sequence).

31. The method of claim 30 wherein said antibody comprises a V_H sequence selected from the group consisting of amino acids 1 to 97 of SEQ ID NO: 11 (HB22-7 V_H sequence); amino acids 1 to 100 of SEQ ID NO: 15 (HB22-23 V_H sequence); and amino acids 1 to 98 of SEQ ID NO: 17 (HB22-33 V_H sequence).

32. The method of claim 1 wherein said antibody comprises a light chain comprising a V_κ sequence having at least about 95 % sequence identity with the amino acid sequence of SEQ ID NO: 21 (HB22-5 V_κ sequence); or SEQ ID NO: 23 (HB22-7 V_κ sequence); or SEQ ID NO: 25 (HB22-13 V_κ sequence); or SEQ ID NO: 27 (HB22-23 V_κ sequence); or SEQ ID NO: 29 (HB22-33 V_κ sequence); or SEQ ID NO: 31 (HB22-196 V_κ sequence).

33. The method of claim 32 wherein said antibody comprises a light chain comprising a V_κ sequence having at least about 95 % sequence identity with the amino acid sequence of SEQ ID NO: 23 (HB22-7 V_κ sequence); or SEQ ID NO: 27 (HB22-23 V_κ sequence); or SEQ ID NO: 29 (HB22-33 V_κ sequence).

34. The method of claim 33 wherein said antibody comprises a V_κ sequence selected from the group consisting of the amino acid sequence of SEQ ID NO: 23 (HB22-7 V_κ sequence); SEQ ID NO: 27 (HB22-23 V_κ sequence); and SEQ ID NO: 29 (HB22-33 V_κ sequence).

35. The method of claim 1 wherein said antibody comprises V_H and V_κ sequences selected from the group consisting of amino acids 1 to 97 of SEQ ID NO: 11 (HB22-7 V_H sequence) and the amino acid sequence of SEQ ID NO: 23 (HB22-7 V_κ sequence); amino acids 1 to 100 of SEQ ID NO: 15 (HB22-23 V_H sequence) and the amino acid sequence of SEQ ID NO: 27 (HB22-23 V_κ sequence); and amino acids 1 to 98 of SEQ ID NO: 17 (HB22-33 V_H sequence) and the amino acid sequence of SEQ ID NO: 29 (HB22-33 V_κ sequence).

36. The method of claim 35 wherein said antibody is chimeric.

37. The method of claim 35 wherein said antibody is humanized.

38. The method of claim 35 wherein said antibody is human.

FIGURE 1

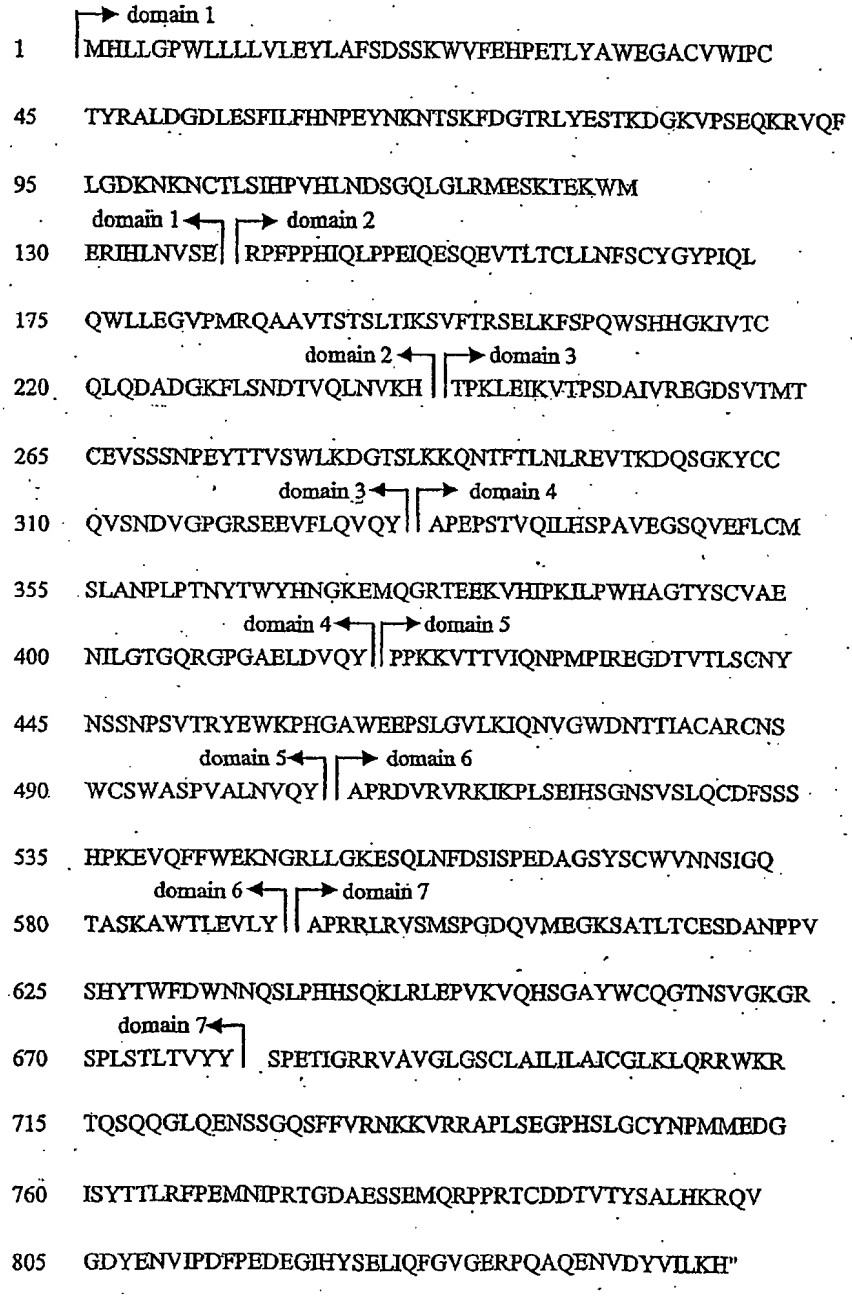

domain 1
1 MHLLGPWLLLLVLEYLAFSDSSKWVFEHPETLYAWEGACVWIPC
45 TYRALDGDLESFILFHNPEYNKNTSKFDGTRLYESTKDGKVPSEQKRVQF
95 LGDKNKNCTLSIHPVHLNDSGQLGLRMESKTEKWM
domain 1
domain 2
130 ERIHLNVSE RPFPPHIQLPPEIQESQEVTLTCLLNFSCYGYPIQL
175 QWLLEGVPMRQAAVTSTSLTIKSVFTRSELKFSPQWSHHGKIVTC
domain 2
domain 3
220 QLQDADGKFLSNDTVQLNVKH TPKLEIKVTPSDAIVREGDSVTMT
265 CEVSSSNPEYTTVSWLKDGTSLKKQNTFTLNLREVTKDQSGKYCC
domain 3
domain 4
310 QVSNDVGPGRSEEVFLQVQY APEPSTVQILHSPAVEGSQVEFLCM
355 SLANPLPTNYTWYHNGKEMQGRTEEKVHIPKILPWHAGTYSCVAE
domain 4
domain 5
400 NILGTGQRGPGAELDVQY PPKKVTTVIQNPMPIREGDTVTLSCNY
445 NSSNPSVTRYEWKPHGAWEEPSLGVLKIQNVGWDNTTIACARCNS
domain 5
domain 6
490 WCSWASPVALNVQY APRDVRVRKIKPLSEIHSGNSVSLQCDFSSS
535 HPKEVQFFWEKNGRLLGKESQLNFDSISPEDAGSYSCWVNNSIGQ
domain 6
domain 7
580 TASKAWTLEVLY APRRLRVSMSPGDQVMEGKSATLTCESDANPPV
625 SHYTWFDWNNQSLPHHSQKLRLEPVKVQHSGAYWCQGTNSVGKGR
domain 7
670 SPLSTLTVYY SPETIGRRVAVGLGSCLAILILAICGLKLQRRWKR
715 TQSQQGLQENSSGQSFFVRNKKVRRAPLSEGPHSLGCYNPMMEDG
760 ISYTTLRFPEMNIPRTGDAESSEMQRPPRTCDDTVTYSALHKRQV
805 GDYENVIPDFPEDEGIHYSELIQFGVGERPQAQENVDYVILKH"

Figure 2

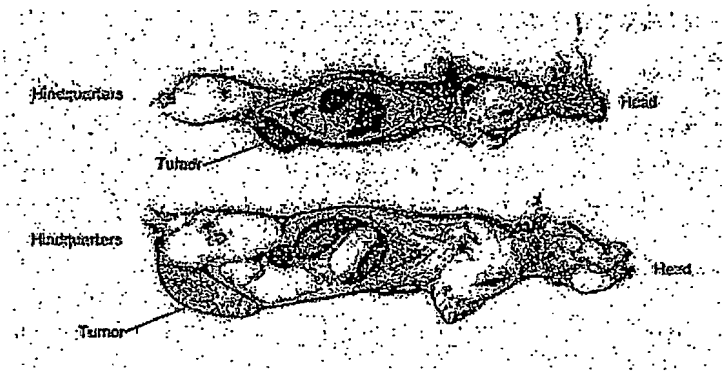


Figure 3

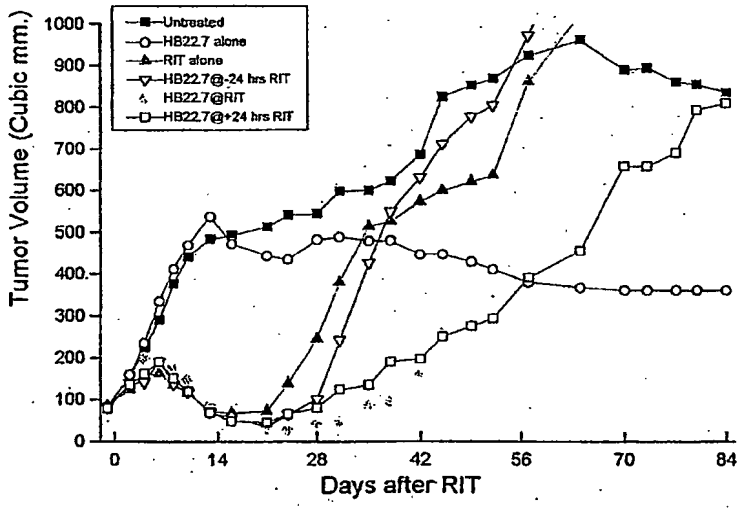
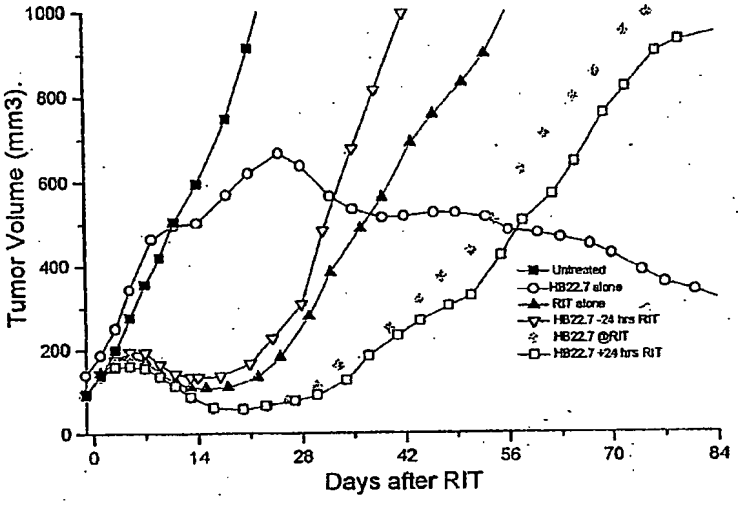

Untreated
HB22.7 alone
RIT alone
HB22.7@-24 hrs RIT
HB22.7@RIT
HB22.7@+24 hrs RIT
Tumor Volume (Cubic mm.)
1000
900
800
700
600
500
400
300
200
100
0
0
14
28
42
56
70
84
Days after RIT

Figure 4


Tumor Volume (mm3)
1000
800
600
400
200
0
0
14
28
42
56
70
84
Days after RIT
Untreated
HB22.7 alone
RIT alone
HB22.7 -24 hrs RIT
HB22.7 @RIT
HB22.7 +24 hrs RIT

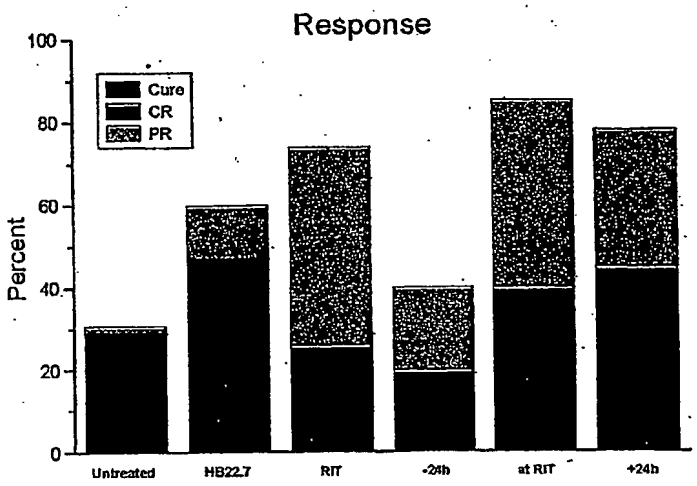

Response
Cure
CR
PR
Percent
100
80
60
40
20
0
Untreated
HB22.7
RIT
-24h
at RIT
+24h

Figure 5

Figure 6

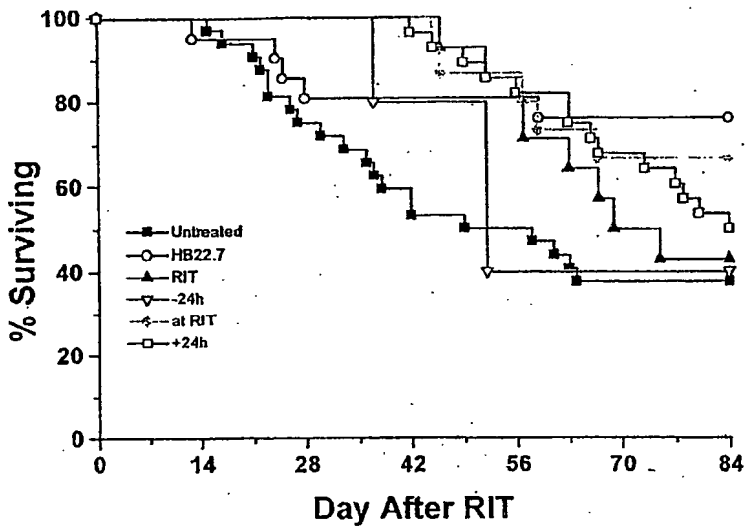
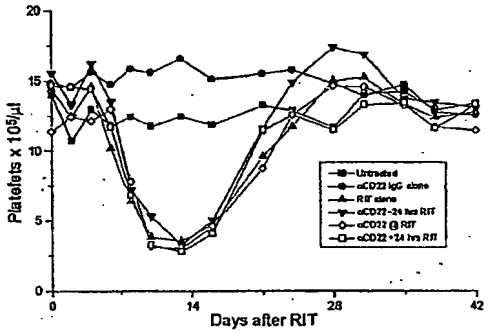

100
80
60
40
20
0
% Surviving
Untreated
HB22.7
RIT
-24h
at RIT
+24h
0
14
28
42
56
70
84
Day After RIT

Figure 7


Platelets x 10⁵/µl
Days after RIT
0
5
10
15
20
0
14
28
42

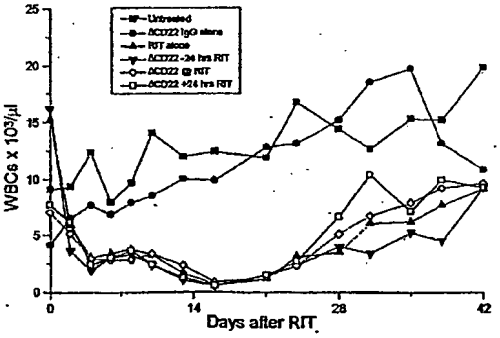

WBCs x 10³/µl
Days after RIT
0
5
10
15
20
25
0
14
28
42

Figure 8

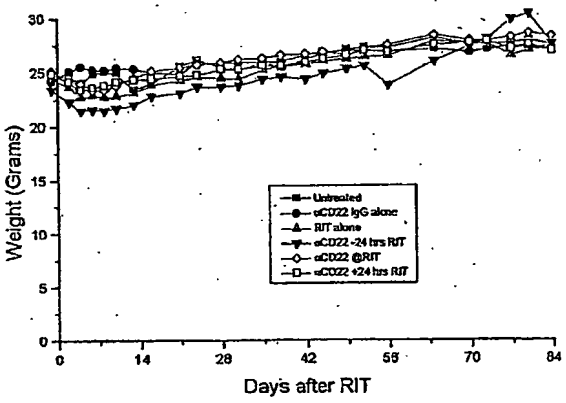

Weight (Grams)
Days after RIT
0
5
10
15
20
25
30
0
14
28
42
56
70
84
Untreated
αCD22 IgG alone
RIT alone
αCD22 -24 hrs RIT
αCD22 @RIT
αCD22 +24 hrs RIT

Figure 9

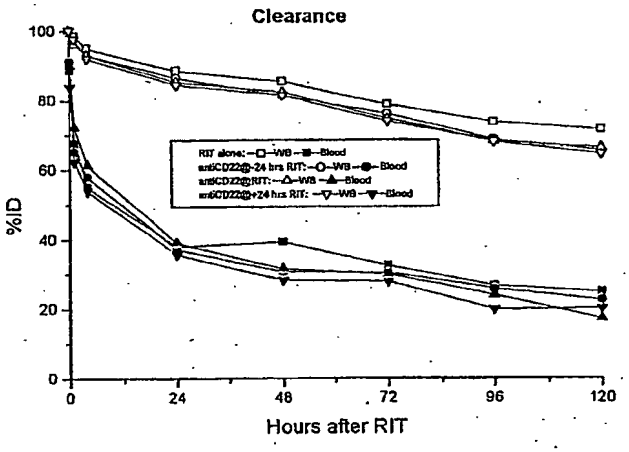


Figure 10

HB22 Hybridoma Antibody Heavy Chair Sequences

Residue	10	20	30 CDR1	40	50 CDR2	60	70	80	90	94	CDR3
HB22-5	GPELVKPGASMKISCKASGYSF.		TDYTMNWVKQSHGKNLEWIGLLH.		PFNG.GTSYNQKFKGKATLSVDKSSSTAFMELLSLTSEDSAVYFCAR						GTGRN YAMDYWGQGTSVTVSS
HB22-196	GPDLVKPGASVKISCKASGYSF.		IGYYMHWLKQSHGKSLEWIGRVN.		PNTA.GLTYNQRFKDKAILTVDKSSNTAYMELRSLTSEDSAVYYCSR						VDYDDYG YWFFDVWGAGTTVTVSS
HB22-7	GPGLVAPSQSLSITCTVSGFSL.		SDYGVNWVRQIPGKGLEWLGIIW.		.GDG.RTDYNSALKSRLNISKDNSKSQVFLKMNSLKADDTARYYCAR						APGNR AMEYWGQGTSVTVSS
HB22-33	GPGLVKPSQSLSLTCSVTGYSITSGYYWNWIRQFPGNKLEWMGYIR.				.YDG.SNNYNPSLKNRISITRQTSKNQFFLKLNSVTTEDTATYYCAR						GGITV AMDYWGQGTSVTVSS
HB22-13	GGGLVQPGGSLRLSCATSGFTF.		IDYYMNWVRQPPGKALEWLGFIKNKFNGYTTEYNTSVKGRFTISRDNSQSILYLQMNTLRAEDSATYYCAR								GLGRS YAMDYWGQGTSVTVSS
HB22-23	GGGLGATWRSMKLSCVASGFTF.		SYYWMNWVRQSPEKGLEWIAEIRLKSNNYATHYAESVKGRFTISRDDSKSSVYLQMNNLRAEDTGIYYCTR								YDGSSR DYWGQGTTLTVSS
			V_k Sequences								D Region J Sequences

Figure 11

HB22-5 VH Sequence

```
 1                                  10                                  20
 E   V   Q   L   Q   E   S   G   P   E   L   V   K   P   G   A   S   M   K   I
GAG GTG CAG CTG CAG GAG TCT GGA CCT GAG CTG GTG AAG CCT GGA GCT TCA ATG AAG ATA   60

 21                                 30                                  40
 S   C   K   A   S   G   Y   S   F   T   D   Y   T   M   N   W   V   K   Q   S
TCC TGC AAG GCT TCT GGT TAC TCA TTC ACT GAC TAC ACC ATG AAC TGG GTG AAG CAG AGC  120

 41                                 50                                  60
 H   G   K   N   L   E   W   I   G   L   L   H   P   F   N   G   G   T   S   Y
CAT GGA AAG AAC CTT GAG TGG ATT GGA CTT CTT CAT CCT TTC AAT GGT GGT ACT AGC TAC  180

 61                                 70                                  80
 N   Q   K   F   K   G   K   A   T   L   S   V   D   K   S   S   S   T   A   F
AAC CAG AAG TTC AAG GGC AAG GCC ACA TTA TCT GTA GAC AAG TCA TCC AGC ACA GCC TTC  240

 81                                 90                                 100
 M   E   L   L   S   L   T   S   E   D   S   A   V   Y   F   C   A   R   G   T
ATG GAG CTC CTC AGT CTG ACA TCT GAG GAC TCT GCA GTC TAT TTC TGT GCA AGA GGG ACA  300

101                                110                                120
 G   R   N   Y   A   M   D   Y   W   G   Q   G   T   S   V   T   V   S   S
GGT CGG AAC TAT GCT ATG GAC TAC TGG GGT CAA GGA ACC TCA GTC ACC GTC TCC TCA      357
```

Figure 12

HB22-7 VH Sequence

```
1                                 10                                  20
E  V  Q  L  Q  E  S  G  P  G  L  V  A  P  S  Q  S  L  S  I
GAG GTG CAG CTG CAG GAG TCT GGA CCT GGC CTG GTG GCG CCC TCA CAG AGC CTG TCC ATC   60

21                                30                                  40
T  C  T  V  S  G  F  S  L  S  D  Y  G  V  N  W  V  R  Q  I
ACA TGC ACC GTC TCA GGG TTC TCA TTA AGC GAC TAT GGT GTA AAC TGG GTT CGC CAG ATT  120

41                                50                                  60
P  G  K  G  L  E  W  L  G  I  I  W  G  D  G  R  T  D  Y  N
CCA GGA AAG GGT CTG GAG TGG CTG GGA ATA ATA TGG GGT GAT GGA AGG ACA GAC TAT AAT  180

61                                70                                  80
S  A  L  K  S  R  L  N  I  S  K  D  N  S  K  S  Q  V  F  L
TCA GCT CTC AAA TCC AGA CTG AAC ATC AGC AAG GAC AAC TCC AAG AGC CAA GTT TTC TTG  240

81                                90                                 100
K  M  N  S  L  K  A  D  D  T  A  R  Y  Y  C  A  R  A  P  G
AAA ATG AAC AGT CTG AAA GCT GAT GAC ACA GCC AGG TAC TAC TGT GCC AGA GCC CCC GGT  300

101                              110                          117
N  R  A  M  E  Y  W  G  Q  G  T  S  V  T  V  S  S
AAT AGG GCT ATG GAG TAC TGG GGT CAA GGA ACC TCA GTC ACC GTC TCC TCA              351
```

Figure 13

HB22-13.VH Sequence

```
1                                   10                                  20
 E   V   Q   L   Q   E   S   G   G   G   L   V   Q   P   G   G   S   L   R   L
GAG GTG CAG CTG CAG GAG TCT GGA GGA GGC TTG GTA CAG CCT GGG GGT TCT CTG AGA CTC   60

21                                  30                                  40
 S   C   A   T   S   G   F   T   F   I   D   Y   Y   M   N   W   V   R   Q   P
TCC TGT GCA ACT TCT GGG TTC ACC TTC ATT GAT TAC TAC ATG AAC TGG GTC CGC CAG CCT  120

41                                  50                                  60
 P   G   K   A   L   E   W   L   G   F   I   K   N   K   F   N   G   Y   T   T
CCA GGA AAG GCA CTT GAG TGG TTG GGT TTT ATT AAA AAC AAA TTT AAT GGT TAC ACA ACA  180

61                                  70                                  80
 E   Y   N   T   S   V   K   G   R   F   T   I   S   R   D   N   S   Q   S   I
GAA TAC AAT ACA TCT GTG AAG GGT CGG TTC ACC ATC TCC AGA GAT AAT TCC CAA AGC ATC  240

81                                  90                                  100
 L   Y   L   Q   M   N   T   L   R   A   E   D   S   A   T   Y   Y   C   A   R
CTC TAT CTT CAA ATG AAC ACC CTG AGA GCT GAG GAC AGT GCC ACT TAT TAC TGT GCA AGA  300

101                                 110                                 120
 G   L   G   R   S   Y   A   M   D   Y   W   G   Q   G   T   S   V   T   V   S
GGG CTG GGA CGT AGC TAT GCT ATG GAC TAC TGG GGT CAA GGA ACC TCA GTC ACC GTC TCC  360

121
 S
TCA                                                                          363
```

Figure 14

HB22-23 VH Sequence

```
1                                   10                                  20
E   V   Q   L   Q   E   S   G   G   G   L   G   A   T   W   R   S   M   K   L
GAG GTG CAG CTG CAG GAG TCT GGA GGA GGG CTT GGT GCA ACC TGG AGA TCC ATG AAA CTC   60

21                                  30                                  40
S   C   V   A   S   G   F   T   F   S   Y   Y   W   M   N   W   V   R   Q   S
TCC TGT GTT GCC TCT GGA TTC ACT TTC AGT TAC TAC TGG ATG AAC TGG GTC CGC CAG TCT  120

41                                  50                                  60
P   E   K   G   L   E   W   I   A   E   I   R   L   K   S   N   N   Y   A   T
CCA GAG AAG GGG CTT GAG TGG ATT GCT GAA ATT AGA TTG AAA TCT AAT AAT TAT GCA ACA  180

61                                  70                                  80
H   Y   A   E   S   V   K   G   R   F   T   I   S   R   D   D   S   K   S   S
CAT TAT GCG GAG TCT GTG AAA GGG AGG TTC ACC ATC TCA AGA GAT GAT TCC AAA AGT AGT  240

81                                  90                                  100
V   Y   L   Q   M   N   N   L   R   A   E   D   T   G   I   Y   Y   C   T   R
GTC TAC CTG CAA ATG AAC AAC TTA AGA GCT GAA GAC ACT GGC ATT TAT TAC TGT ACC AGG  300

101                                 110                                 120
Y   D   G   S   S   R   D   Y   W   G   Q   G   T   T   L   T   V   S   S
TAT GAT GGT TCC TCC CGG GAC TAC TGG GGC CAA GGC ACC ACT CTC ACA GTC TCC TCA      357
```

Figure 15

HB22-33 VH Sequence

```
1                                   10                                  20
E   V   Q   L   Q   E   S   G   P   G   L   V   K   P   S   Q   S   L   S   L
GAG GTG CAG CTG CAG GAG TCT GGA CCT GGC CTC GTG AAA CCT TCT CAG TCT CTG TCT CTC   60

21                                  30                                  40
T   C   S   V   T   G   Y   S   I   T   S   G   Y   Y   W   N   W   I   R   Q
ACC TGC TCT GTC ACT GGC TAC TCC ATC ACC AGT GGT TAT TAC TGG AAC TGG ATC CGG CAG  120

41                                  50                                  60
F   P   G   N   K   L   E   W   M   G   Y   I   R   Y   D   G   S   N   N   Y
TTT CCA GGA AAC AAA CTG GAA TGG ATG GGC TAC ATT AGG TAC GAC GGT AGC AAT AAC TAC  180

61                                  70                                  80
N   P   S   L   K   N   R   I   S   I   T   R   D   T   S   K   N   Q   F   F
AAC CCA TCT CTC AAA AAT CGA ATC TCC ATC ACT CGT GAC ACA TCT AAG AAC CAG TTT TTC  240

81                                  90                                  100
L   K   L   N   S   V   T   T   E   D   T   A   T   Y   Y   C   A   R   G   G
CTG AAG TTG AAT TCT GTG ACT ACT GAG GAC ACA GCT ACA TAT TAC TGT GCA AGA GGG GGG  300

101                                 110                 118
I   T   V   A   M   D   Y   W   G   Q   G   T   S   V   T   V   S   S
ATT ACG GTT GCT ATG GAC TAC TGG GGT CAA GGA ACC TCA GTC ACC GTC TCC TCA          360
```

Figure 16

HB22-196 VH Sequence

```
 1                                  10                                  20
 E   V   Q   L   Q   E   S   G   P   D   L   V   K   P   G   A   S   V   K   I
GAG GTG CAG CTG CAG GAG TCT GGA CCT GAC CTG GTG AAG CCT GGG GCT TCA GTG AAG ATA   60

 21                                 30                                  40
 S   C   K   A   S   G   Y   S   F   I   G   Y   Y   M   H   W   L   K   Q   S
TCC TGT AAG GCT TCT GGT TAC TCA TTC ATT GGC TAT TAC ATG CAC TGG CTG AAG CAG AGC  120

 41                                 50                                  60
 H   G   K   S   L   E   W   I   G   R   V   N   P   N   T   A   G   L   T   Y
CAT GGA AAG AGC CTT GAG TGG ATT GGA GCT GTT AAT CCT AAC ACT GCT GGT CIT ACC TAC  180

 61                                 70                                  80
 N   Q   R   F   K   D   K   A   I   L   T   V   D   K   S   S   N   T   A   Y
AAC CAG AGG TTC AAG GAC AAG GCC ATA TTA ACT GTA GAC AAG TCA TCC AAC ACA GCC TAT  240

 81                                 90                                  100
 M   E   L   R   S   L   T   S   E   D   S   A   V   Y   Y   C   S   R   V   D
ATG GAG CTC CGC AGC CTG ACA TCT GAG GAC TCT GCG GTC TAT TAC TGT TCA AGA GTG GAC  300

101                                110                                 120
 Y   D   D   Y   G   Y   W   F   F   D   V   W   G   A   G   T   T   V   T   V
TAT GAT GAC TAC GGG TAC TGG TTC TTC GAT GTC TGG GGC GCA GGG ACC ACG GTC ACC GTC  360

121
 S   S
TCC TCA                                                                    366
```

Figure 17

HB22 Hybridoma Antibody V kappa light chain sequences

Antibody	-20	-10	1	10	20	CDR1 ABCDEFG	35	40	50	CDR2	60	70	80	90	CDR3	J Segment	Constant
HB22-5	MESQTQVFVFLLLCVSGAHGSIVMTQTPKFLLVSTGDRVTITCKASQTVT						NDLAWYQQKPGQSPKLLIYYASNRYTGVPDRFTGSGYGTDFTFTINTVQAEDLAVYFCQQDYSSP									LTFGAGTKLELK	RADAAPTV
HB22-7	MESQTQVFVFLLLCVSGAHGSIVMTQTPKFLLVSAGDRITLTCKASQSVT						NDVAWYQQKPGQSPKLLIYYASNRYTGVPDRFTGSGYGTDFTFTISTVQAEDLAVYFCQQDYRSP									WTFGGGTKLEIK	RADAAPTV
HB22-13	MESQTQVFVFLLLCVSGAHGSIVMTQTPKFLLVSAGDRVSITCKASQSVT						NDVTWYQQKPGQSPKLLIYFASNRYTGVPDRFTGSGYGTDFTFTISTVQAEDLAVYFCQQDYSSP									LTFGAGTKLELK	RADAAPTV
HB22-23	MESQTQVFVFLLLCVSGAHGSIVMTQTPKFLLVSAGDRVSITCKASQSVT						NDVTWYQQKPGQSPKLLIYFASNRYTGVPDRFTGSGYGTDFTFTISTVQAEDLAVYFCQQDYSSP									LTFGAGTKLELK	RADAAPTV
HB22-33	MKLPVRLLVLMFWIPASSSDVVMTQTPLSLPVSLGDQASISCRSSQSLVHSNGNTYLHWYLQKPGQSPKLLIYKVSNRFSGVPDRFSGSGSGTDFTLKISRVEAEDLGVYFCSQSTHVP															YTFGGGTKLEIK	RADAAPTV
HB22-196	MESQTQVFISILLWLYGADGNIVMTQSPKSMSMSVGERVTLTCKASENVV						TYVSWYQQKPEQSPKLLIYGASNRYTGVPDRFTGSGSATDFTLTISSVQAEDLADYHCGQGYSYP									YTFGGGTKLEIK	RADAAPTV

Figure 18

HB22-5 Vk Sequence

```
    M  E  S  Q  T  Q  V  F  V  F  L  L  L  C  V  S  G  A  H
AAG ATG GAG TCA CAG ACC CAG GTC TTC GTA TTT CTA CTG CTC TGT GTG TCT GGT GCT CAT      60

 G  S  I  V  M  T  Q  T  P  K  F  L  L  V  S  T  G  D  R  V
GGG AGT ATT GTG ATG ACC CAG ACT CCC AAA TTC CTG CTT GTA TCA ACA GGA GAC AGG GTT     120

 T  I  T  C  K  A  S  Q  T  V  T  N  D  L  A  W  Y  Q  Q  K
ACC ATT ACC TGC AAG GCC AGT CAG ACT GTG ACT AAT GAT TTA GCT TGG TAC CAA CAG AAG     180

 P  G  Q  S  P  K  L  L  I  Y  Y  A  S  N  R  Y  T  G  V  P
CCA GGG CAG TCT CCT AAA CTG CTG ATA TAC TAT GCA TCC AAT CGC TAC ACT GGA GTC CCT     240

 D  R  F  T  G  S  G  Y  G  T  D  F  T  F  T  I  N  T  V  Q
GAT CGC TTC ACT GGC AGT GGA TAT GGG ACG GAC TTC ACT TTC ACC ATC AAC ACT GTG CAG     300

 A  E  D  L  A  V  Y  F  C  Q  Q  D  Y  S  S  P  L  T  F  G
GCT GAA GAC CTG GCA GTT TAT TTC TGT CAG CAG GAT TAT AGC TCT CCT CTC ACG TTC GGT     360

 A  G  T  K  L  E  L  K  R  A  D  A  A  P  T  V
GCT GGG ACC AAG CTG GAA CTG AAA CGG GCT GAT GCT GCA CCA ACT GTA TC                  410
```

Figure 19

HB22-7 Vk Sequence

```
    M  E  S  Q  T  Q  V  F  V  F  L  L  L  C  V  S  G  A  H
AAG ATG GAG TCA CAG ACC CAG GTC TTC GTA TTT CTA CTG CTC TGT GTG TCT GGT GCT CAT      60

 G  S  I  V  M  T  Q  T  P  K  F  L  L  V  S  A  G  D  R  I
GGG AGT ATT GTG ATG ACC CAG ACT CCC AAA TTC CTG CTT GTA TCA GCA GGA GAC AGG ATT     120

 T  L  T  C  K  A  S  Q  S  V  T  N  D  V  A  W  Y  Q  Q  K
ACC TTA ACC TGC AAG GCC AGT CAG AGT GTG ACT AAT GAT GTA GCT TGG TAC CAA CAG AAG     180

 P  G  Q  S  P  K  L  L  I  Y  Y  A  S  N  R  Y  T  G  V  P
CCA GGG CAG TCT CCT AAA CTG CTG ATA TAC TAT GCA TCC AAT CGC TAC ACT GGA GTC CCT     240

 D  R  F  T  G  S  G  Y  G  T  D  F  T  F  T  I  S  T  V  Q
GAT CGC TTC ACT GGC AGT GGA TAT GGG ACG GAT TTC ACT TTC ACC ATC AGC ACT GTG CAG     300

 A  E  D  L  A  V  Y  F  C  Q  Q  D  Y  R  S  P  W  T  F  G
GCT GAA GAC CTG GCA GTT TAT TTC TGT CAG CAG GAT TAT AGG TCT CCG TGG ACG TTC GGT     360

 G  G  T  K  L  E  I  K  R  A  D  A  A  P  T  V
GGA GGC ACC AAG CTG GAA ATC AAA CGG GCT GAT GCT GCA CCA ACT GTA TC                  410
```

Figure 20

HB22-13 Vk Sequence

```
   M  E  S  Q  T  Q  V  F  V  F  L  L  L  C  V  S  G  A  H
AAG ATG GAG TCA CAG ACC CAG GTC TTC GTA TTT CTA CTG CTC TGT GTG TCT GGT GCT CAT      60

 G  S  I  V  M  T  Q  T  P  K  F  L  L  V  S  A  G  D  R  V
GGG AGT ATT GTG ATG ACC CAG ACT CCC AAA TTC CTG CTT GTA TCA GCA GGA GAC AGG GTT     120

 S  I  T  C  K  A  S  Q  S  V  T  N  D  V  T  W  Y  Q  Q  K
TCC ATA ACC TGC AAG GCC AGT CAG AGT GTG ACT AAT GAT GTA ACT TGG TAC CAA CAG AAG     180

 P  G  Q  S  P  K  L  L  I  Y  F  A  S  N  R  Y  T  G  V  P
CCA GGG CAG TCT CCT AAA TTG CTG ATA TAC TTT GCA TCC AAT CGC TAC ACT GGA GTC CCT     240

 D  R  F  T  G  S  G  Y  G  T  D  F  T  F  T  I  S  T  V  Q
GAT CGC TTC ACT GGC AGT GGA TAT GGG ACG GAT TTC ACT TTC ACC ATC AGC ACT GTG CAG     300

 A  E  D  L  A  V  Y  F  C  Q  Q  D  Y  S  S  P  L  T  F  G
GCT GAA GAC CTG GCA GTT TAT TTC TGT CAG CAG GAT TAT AGC TCT CCG CTC ACG TTC GGT     360

 A  G  T  K  L  E  L  K  R  A  D  A  A  P  T  V
GCT GGG ACC AAG CTG GAG CTG AAA CGG GCT GAT GCT GCA CCA ACT GTA TC                  410
```

Figure 21

HB22-23 Vk Sequence

```
    M   E   S   Q   T   Q   V   F   V   F   L   L   L   C   V   S   G   A   H
AAG ATG GAG TCA CAG ACC CAG GTC TTC GTA TTT CTA CTG CTC TGT GTG TCT GGT GCT CAT     50

 G   S   I   V   M   T   Q   T   P   K   F   L   L   V   S   A   G   D   R   V
GGG AGT ATT GTG ATG ACC CAG ACT CCC AAA TTC CTG CTT GTA TCA GCA GGA GAC AGG GTC    100

 T   I   S   C   K   A   S   Q   S   V   S   N   D   V   A   W   Y   Q   Q   K
ACC ATA AGC TGC AAG GCC AGT CAG AGT GTG AGT AAT GAT GTA GCT TGG TAC CAA CAG AAG    150

 P   G   Q   S   P   K   L   L   I   Y   Y   A   S   K   R   Y   T   G   V   P
CCA GGG CAG TCT CCT AAA CTG CTG ATA TAC TAT GCA TCC AAG CGC TAT ACT GGA GTC CCT    200

 D   R   L   T   G   S   G   Y   G   T   D   F   T   F   T   I   S   T   V   Q
GAT CGC CTC ACT GGC AGT GGA TAT GGG ACG GAT TTC ACT TTC ACC ATC AGC ACT GTG CAG    250

 A   E   D   L   A   V   Y   F   C   Q   Q   D   H   S   Y   P   W   T   F   G
GCT GAA GAC CTG GCA GTT TAT TTC TGT CAG CAG GAT CAT AGC TAT CCG TGG ACG TTC GGT    300

 G   G   T   K   L   E   I   K   R   A   D   A   A   P   T   V
GGA GGC ACC AAG CTG GAG ATC AAA CGG GCT GAT GCT GCA CCA ACT GTA TC                 350
```

Figure 22

HB22-33 Vk Sequence

```
 M   K   L   P   V   R   L   L   V   L   M   F   W   I   P   A   S   S   S   D
ATG AAG TTG CCT GTT AGG CTG TTG GTG CTG ATG TTC TGG ATT CCT GCT TCC AGC AGT GAT    60

 V   V   M   T   Q   T   P   L   S   L   P   V   S   L   G   D   Q   A   S   I
GTT GTG ATG ACC CAA ACT CCA CTC TCC CTG CCT GTC AGT CTT GGA GAT CAA GCC TCC ATC   120

 S   C   R   S   S   Q   S   L   V   H   S   N   G   N   T   Y   L   H   W   Y
TCT TGC AGA TCT AGT CAG AGC CTT GTA CAC AGT AAT GGA AAC ACC TAT TTA CAT TGG TAC   180

 L   Q   K   P   G   Q   S   P   K   L   L   I   Y   K   V   S   N   R   F   S
CTG CAG AAG CCA GGC CAG TCT CCA AAG CTC CTG ATC TAC AAA GTT TCC AAC CGA TTT TCT   240

 G   V   P   D   R   F   S   G   S   G   S   G   T   D   F   T   L   K   I   S
GGG GTC CCA GAT AGG TTC AGT GGC AGT GGA TCA GGG ACA GAT TTC ACA CTC AAG ATC AGC   300

 R   V   E   A   E   D   L   G   V   Y   F   C   S   Q   S   T   H   V   P   Y
AGA GTG GAG GCT GAG GAT CTG GGA GTT TAT TTC TGC TCT CAA AGT ACA CAT GTT CCG TAC   360

 T   F   G   G   G   T   K   L   E   I   K   R   A   D   A   A   P   T   V
ACG TTC GGA GGG GGG ACC AAG CTG GAA ATA AAA CGG GCT GAT GCT GCA CCA ACT GTA TC    419
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Figure; 23

HB22-196 Vk Sequence

```
    M  E  S  Q  T  Q  V  F  I  S  I  L  L  W  L  Y  G  A  D
AAG ATG GAG TCA CAG ACC CAG GTC TTC ATA TCC ATA CTG CTC TGG TTA TAT GGA GCT GAT      60

 G  N  I  V  M  T  Q  S  P  K  S  M  S  M  S  V  G  E  R  V
GGG AAC ATT GTA ATG ACC CAA TCT CCC AAA TCC ATG TCC ATG TCA GTA GGA GAG AGG GTC     120

 T  L  T  C  K  A  S  E  N  V  V  T  Y  V  S  W  Y  Q  Q  K
ACC TTG ACC TGC AAG GCC AGT GAG AAT GTG GTT ACT TAT GTT TCC TGG TAT CAA CAG AAA     180

 P  E  Q  S  P  K  L  L  I  Y  G  A  S  N  R  Y  T  G  V  P
CCA GAG CAG TCT CCT AAA CTG CTG ATA TAC GGG GCA TCC AAC CGG TAC ACT GGG GTC CCC     240

 D  R  F  T  G  S  G  S  A  T  D  F  T  L  T  I  S  S  V  Q
GAT CGC TTC ACA GGC AGT GGA TCT GCA ACA GAT TTC ACT CTG ACC ATC AGC AGT GTG CAG     300

 A  E  D  L  A  D  Y  H  C  G  Q  G  Y  S  Y  P  Y  T  F  G
GCT GAA GAC CTT GCA GAT TAT CAC TGT GGA CAG GGT TAC AGC TAT CCG TAC ACG TTC GGA     360

 G  G  T  K  L  E  I  K  R  A  D  A  A  P  T  V
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SEQUENCE LISTING

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TUSCANO, Joseph
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Tyr Ala Trp Glu Gly Ala Cys Val Trp Ile Pro Cys Thr Tyr Arg Ala
        35                  40                  45
Leu Asp Gly Asp Leu Glu Ser Phe Ile Leu Phe His Asn Pro Glu Tyr
    50                  55                  60
Asn Lys Asn Thr Ser Lys Phe Asp Gly Thr Arg Leu Tyr Glu Ser Thr
65                  70                  75                  80
Lys Asp Gly Lys Val Pro Ser Glu Gln Lys Arg Val Gln Phe Leu Gly
                85                  90                  95
Asp Lys Asn Lys Asn Cys Thr Leu Ser Ile His Pro Val His Leu Asn
            100                 105                 110
Asp Ser Gly Gln Leu Gly Leu Arg Met Glu Ser Lys Thr Glu Lys Trp
        115                 120                 125
Met Glu Arg Ile His Leu Asn Val Ser Glu Arg Pro Phe Pro Pro His
    130                 135                 140
Ile Gln Leu Pro Pro Glu Ile Gln Glu Ser Gln Glu Val Thr Leu Thr
145                 150                 155                 160
Cys Leu Leu Asn Phe Ser Cys Tyr Gly Tyr Pro Ile Gln Leu Gln Trp
                165                 170                 175
Leu Leu Glu Gly Val Pro Met Arg Gln Ala Ala Val Thr Ser Thr Ser
            180                 185                 190
Leu Thr Ile Lys Ser Val Phe Thr Arg Ser Glu Leu Lys Phe Ser Pro
        195                 200                 205
Gln Trp Ser His His Gly Lys Ile Val Thr Cys Gln Leu Gln Asp Ala
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Asp Gly Lys Phe Leu Ser Asn Asp Thr Val Gln Leu Asn Val Lys His
225                 230                 235                 240
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            260                 265                 270
Glu Tyr Thr Thr Val Ser Trp Leu Lys Asp Gly Thr Ser Leu Lys Lys
        275                 280                 285
Gln Asn Thr Phe Thr Leu Asn Leu Arg Glu Val Thr Lys Asp Gln Ser
    290                 295                 300
Gly Lys Tyr Cys Cys Gln Val Ser Asn Asp Val Gly Pro Gly Arg Ser
305                 310                 315                 320
Glu Glu Val Phe Leu Gln Val Gln Tyr Ala Pro Glu Pro Ser Thr Val
                325                 330                 335
Gln Ile Leu His Ser Pro Ala Val Glu Gly Ser Gln Val Glu Phe Leu
            340                 345                 350
Cys Met Ser Leu Ala Asn Pro Leu Pro Thr Asn Tyr Thr Trp Tyr His
        355                 360                 365
Asn Gly Lys Glu Met Gln Gly Arg Thr Glu Glu Lys Val His Ile Pro
    370                 375                 380
Lys Ile Leu Pro Trp His Ala Gly Thr Tyr Ser Cys Val Ala Glu Asn
385                 390                 395                 400
Ile Leu Gly Thr Gly Gln Arg Gly Pro Gly Ala Glu Leu Asp Val Gln
                405                 410                 415
Tyr Pro Pro Lys Lys Val Thr Thr Val Ile Gln Asn Pro Met Pro Ile
            420                 425                 430
Arg Glu Gly Asp Thr Val Thr Leu Ser Cys Asn Tyr Asn Ser Ser Asn
        435                 440                 445
Pro Ser Val Thr Arg Tyr Glu Trp Lys Pro His Gly Ala Trp Glu Glu
    450                 455                 460
Pro Ser Leu Gly Val Leu Lys Ile Gln Asn Val Gly Trp Asp Asn Thr
465                 470                 475                 480
Thr Ile Ala Cys Ala Arg Cys Asn Ser Trp Cys Ser Trp Ala Ser Pro
                485                 490                 495
Val Ala Leu Asn Val Gln Tyr Ala Pro Arg Asp Val Arg Val Arg Lys
            500                 505                 510
Ile Lys Pro Leu Ser Glu Ile His Ser Gly Asn Ser Val Ser Leu Gln
        515                 520                 525
Cys Asp Phe Ser Ser Ser His Pro Lys Glu Val Gln Phe Phe Trp Glu
    530                 535                 540
Lys Asn Gly Arg Leu Leu Gly Lys Glu Ser Gln Leu Asn Phe Asp Ser
545                 550                 555                 560
Ile Ser Pro Glu Asp Ala Gly Ser Tyr Ser Cys Trp Val Asn Asn Ser
                565                 570                 575
Ile Gly Gln Thr Ala Ser Lys Ala Trp Thr Leu Glu Val Leu Tyr Ala
            580                 585                 590
Pro Arg Arg Leu Arg Val Ser Met Ser Pro Gly Asp Gln Val Met Glu
        595                 600                 605
Gly Lys Ser Ala Thr Leu Thr Cys Glu Ser Asp Ala Asn Pro Pro Val
    610                 615                 620
Ser His Tyr Thr Trp Phe Asp Trp Asn Asn Gln Ser Leu Pro His His
625                 630                 635                 640
Ser Gln Lys Leu Arg Leu Glu Pro Val Lys Val Gln His Ser Gly Ala
                645                 650                 655
Tyr Trp Cys Gln Gly Thr Asn Ser Val Gly Lys Gly Arg Ser Pro Leu
            660                 665                 670
Ser Thr Leu Thr Val Tyr Tyr Ser Pro Glu Thr Ile Gly Arg Arg Val
        675                 680                 685
Ala Val Gly Leu Gly Ser Cys Leu Ala Ile Leu Ile Leu Ala Ile Cys
    690                 695                 700
Gly Leu Lys Leu Gln Arg Arg Trp Lys Arg Thr Gln Ser Gln Gln Gly
705                 710                 715                 720
Leu Gln Glu Asn Ser Ser Gly Gln Ser Phe Phe Val Arg Asn Lys Lys
                725                 730                 735
Val Arg Arg Ala Pro Leu Ser Glu Gly Pro His Ser Leu Gly Cys Tyr
            740                 745                 750
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785                 790                 795                 800
Lys Arg Gln Val Gly Asp Tyr Glu Asn Val Ile Pro Asp Phe Pro Glu
                805                 810                 815
Asp Glu Gly Ile His Tyr Ser Glu Leu Ile Gln Phe Gly Val Gly Glu
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catggaaaga accttgagtg gattggactt cttcatcctt tcaatggtgg tactagctac 180
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atggagctcc tcagtctgac atctgaggac tctgcagtct atttctgtgc aagagggaca 300
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35 40 45
Gly Leu Leu His Pro Phe Asn Gly Gly Thr Ser Tyr Asn Gln Lys Phe
50 55 60
Lys Gly Lys Ala Thr Leu Ser Val Asp Lys Ser Ser Ser Thr Ala Phe
65 70 75 80
Met Glu Leu Leu Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys
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35 40 45
Gly Ile Ile Trp Gly Asp Gly Arg Thr Asp Tyr Asn Ser Ala Leu Lys
50 55 60
Ser Arg Leu Asn Ile Ser Lys Asp Asn Ser Lys Ser Gln Val Phe Leu

65 70 75 80
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Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Gln Ser Ile
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35 40 45
Ala Glu Ile Arg Leu Lys Ser Asn Asn Tyr Ala Thr His Tyr Ala Glu
50 55 60
Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asp Ser Lys Ser Ser
65 70 75 80
Val Tyr Leu Gln Met Asn Asn Leu Arg Ala Glu Asp Thr Gly Ile Tyr
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(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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4 September 2003 (04.09.2003)

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(10) International Publication Number
WO 2003/072036 A3

(51) International Patent Classification[7]: A61K 39/395

(21) International Application Number:
PCT/US2003/005323

(22) International Filing Date: 20 February 2003 (20.02.2003)

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(26) Publication Language: English

(30) Priority Data:

60/359,419	21 February 2002 (21.02.2002)	US
60/420,472	21 October 2002 (21.10.2002)	US

(63) Related by continuation (CON) or continuation-in-part (CIP) to earlier application:
US Not furnished (CIP)
Filed on Not furnished

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI (utility model), FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

(88) Date of publication of the international search report:
31 December 2003

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 2003/072036 A3

(54) Title: TREATMENT METHODS USING ANTI-CD22 ANTIBODIES

(57) Abstract: The invention concerns treatment methods using anti-CD22 monoclonal antibodies with unique physiologic properties. In particular, the invention concerns methods for the treatment of B-cell malignancies by administering an effective amount of a blocking anti-CD22 monoclonal antibody specifically binding to the first two Ig-like domains, or to an epitope within the first two Ig-like domains of native human CD22 (hCD22).

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US03/05323

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : A61K 39/395
US CL : 424/130.1, 133.1, 135.1, 136.1, 139.1, 143.1, 144.1, 153.1, 156.1, 174.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 424/130.1, 133.1, 135.1, 136.1, 139.1, 143.1, 144.1, 153.1, 156.1, 174.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5484892 A (TEDDER et al.) 16 January 1996, see entire document.	1-38

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
08 May 2003 (08.05.2003)

Date of mailing of the international search report
08 SEP 2003

Name and mailing address of the ISA/US
Mail Stop PCT, Attn: ISA/US
Commissioner for Patents
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Facsimile No. (703)305-3230

Authorized officer
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Telephone No. 703 3080196

Janice Ford

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

PCT/US03/05323

Continuation of B. FIELDS SEARCHED Item 3:
WEST 2.1, MEDICINE/BIOTECH (compendium databases on DIALOG) search terms: inventor names, cd22, anticd22, hcd22, hb22?, b cell, lymphoma, myeloma, leukemia, cancer, tumor

(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 1 481 992 A2

(12) **EUROPEAN PATENT APPLICATION**

(43) Date of publication:
01.12.2004 Bulletin 2004/49

(21) Application number: **04011466.2**

(22) Date of filing: **26.02.2001**

(51) Int Cl.[7]: **C07K 16/18**, C12N 15/13, C12N 5/10, A61K 39/00, A61P 25/28

(84) Designated Contracting States:
AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU MC NL PT SE TR
Designated Extension States:
AL LT LV MK RO SI

(30) Priority: **24.02.2000 US 184601 P**
08.12.2000 US 254465 P
08.12.2000 US 254498 P

(62) Document number(s) of the earlier application(s) in accordance with Art. 76 EPC:
01913081.4 / 1 257 584

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  St. Louis MO 63110 (US)
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  Cloverdale IN 46120 (US)
- **Paul, Steven, M.**
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  Palo Alto CA 94306 (US)
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  Palo Alto CA 94303 (US)

(74) Representative: **Grund, Martin, Dr. et al**
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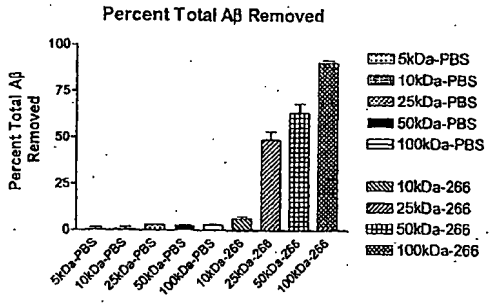
Geibelstrasse 6
81679 München (DE)

Remarks:
This application was filed on 13 - 05 - 2004 as a divisional application to the application mentioned under INID code 62. The sequence listing, which is published as annex to the application documents, was filed after the date of filing. The applicant has declared that it does not include matter which goes beyond the content of the application as filed.

(54) **Humanized antibodies that sequester amyloid beta peptide**

(57) A method to treat conditions characterized by formation of amyloid plaques both prophylactically and therapeutically is described. The method employs humanized antibodies which sequester soluble Aβ peptide from human biological fluids or which preferably specifically bind an epitope contained within position 13-28 of the amyloid beta peptide Aβ.



842

Figure 1

EP 1 481 992 A2

Description

Cross-Reference to Related Applications

[0001] This application claims the priority of, United States provisional applications 60/184,601 filed 24 February 2000,60/254,465, filed 8 December 2000, and 60/254,498, filed 8 December 2000, the contents of each of which are incorporated herein by reference.

Technical Field

[0002] The invention relates to humanized antibodies that bind to an epitope between amino acids 13 and 28 of the Aβ peptide and to preventive and therapeutic treatment of conditions associated with beta amyloid, such as Alzheimer's disease, Down's syndrome, and cerebral amyloid angiopathey. More specifically, it concerns use of humanized monoclonal antibodies to sequester amyloid beta (Aβ) peptide in plasma, brain, and cerebrospinal fluid to prevent accumulation or to reverse deposition of the Aβ peptide within the brain and in the cerebrovasculature and to improve cognition.

Background Art

[0003] A number of symptomologies which result in cognitive deficits, stroke, brain hemorrhage, and general mental debilitation appear to be associated with neuritic and cerebrovascular plaques in the brain containing the amyloid beta peptide (Aβ). Among these conditions are both pre-clinical and clinical Alzheimer's disease, Down's syndrome, and pre-clinical and clinical cerebral amyloid angiopathy (CAA). The amyloid plaques are formed from amyloid beta peptides. These peptides circulate in the blood and in the cerebrospinal fluid (CSF), typically in complexed form with lipoproteins. The Aβ peptide in circulating form is composed of 39-43 amino acids (mostly 40 or 42 amino acids) resulting from the cleavage of a common precursor protein, amyloid precursor protein, often designated APP. Some forms of soluble Aβ are themselves neurotoxic and may determine the severity of neurodegeneration and/or cognitive decline (McLean, C. A., *et al., Ann. Neurol.* (1999) 46:860-866; Lambert, M. P., *et al.* (1998) 95:6448-6453; Naslund, J., J. Am. Med. Assoc. (2000) 283:1571).

[0004] Evidence suggests that Aβ can be transported back and forth between brain and the blood (Ghersi-Egea, J-F., *et al., J. Neurochem.* (1996) 67:880-883; Zlokovic, B. V., *et al., Biochem. Biophys. Res. Comm.* (1993) 67: 1034-1040; Shibata M, *et al., J. Clin. Invest.* (2000) 106:1489-1499). Further Aβ in plaques is in an equilibrium with soluble Aβ in the brain and blood (Kawarabayashi T, *et al., J. Neurosci.* (2001) 21:372-381).

[0005] As described in PCT application US00/35681 and U.S. Serial No. 09/153,130 both incorporated herein by reference, total circulating levels of Aβ peptide in CSF are similar in normal individuals and individuals predisposed to exhibit the symptoms of Alzheimer's. However, $A\beta_{42}$ levels are lower on average in individuals with Alzheimer's disease (Nitsch, R M., *et al., Ann. Neurol.* (1995) 37:512-518). It is known that $A\beta_{42}$ is more prone to aggregate than is $A\beta_{40}$, and when this happens, adverse consequences such as Aβ deposition in amyloid plaques, conversion of Aβ to toxic soluble forms, nerve cell damage, and behavioral impairment such as dementia ensue (Golde, T.E., *et al., Biochem. Biophys. Acta.* (2000) 1502:172-187).

[0006] Methods to induce an immune response to reduce amyloid deposits are described in PCT publication WO99/27944 published 10 June 1999. The description postulates that full-length aggregated Aβ peptide would be a useful immunogen. Administration of a Aβ fragment (amino acids 13-28) conjugated to sheep anti-mouse IgG caused no change in cortex amyloid burden, and only one in nine animals that received injections of the Aβ 13-28 fragment-conjugate showed any lymphoproliferation in response to $A\beta_{40}$. The application also indicates that antibodies that specifically bind to Aβ peptide could be used as therapeutic agents. However, this appears to be speculation since the supporting data reflect protocols that involve active immunization using, for example, $A\beta_{42}$. The peptides are supplied using adjuvants and antibody titers formed from the immunization, as well as levels of Aβ peptide and of the precursor peptide, are determined. The publication strongly suggests that Aβ plaque must be reduced in order to alleviate Alzheimer's symptoms, and that cell-mediated processes are required for successful reduction of Aβ plaque.

[0007] WO 99/60024, published 25 November 1999, is directed to methods for amyloid removal using anti-amyloid antibodies. The mechanism, however, is stated to utilize the ability of anti-Aβ antibodies to bind to pre-formed amyloid deposits *(i.e.,* plaques) and result in subsequent local microglial clearance of localized plaques. This mechanism was not proved *in vivo.* This publication further states that to be effective against Aβ plaques, anti-Aβ antibodies must gain access to the brain parenchyma and cross the blood brain barrier.

[0008] Several PCT applications that relate to attempts to control amyloid plaques were published on 7 December 2000. WO 00/72880 describes significant reduction in plaque in cortex and hippocampus in a transgenic mouse model of Alzheimer' disease when treated using N-terminal fragments of Aβ peptides and antibodies that bind to them, but

not when treated with the Aβ 13-28 fragment conjugated to sheep anti-mouse IgG or with an antibody against the 13-28 fragment, antibody 266. The N-terminal directed antibodies were asserted to cross the blood-brain barrier and to induce phagocytosis of amyloid plaques in *in vitro* studies.

[0009] WO 00/72876 has virtually the same disclosure as WO 00/72880 and is directed to immunization with the amyloid fibril components themselves.

[0010] WO 00/77178 describes antibodies that were designed to catalyze the hydrolysis of β-amyloid, including antibodies raised against a mixture of the phenylalanine statine transition compounds Cys-$A\beta_{10-25}$, statine Phe_{19}-Phe_{20} and Cys-$A\beta_{10-25}$ statine Phe_{20}-Ala_{21} and antibodies raised against $A\beta_{10-25}$ having a reduced amide bond between Phe_{19} and Phe_{20}. This document mentions sequestering of Aβ, but this is speculation because it gives no evidence of such sequestering. Further, the document provides no *in vivo* evidence that administration of antibodies causes efflux of Aβ from the central nervous system, interferes with plaque formation, reduces plaque burden, forms complexes between the antibodies and Aβ in tissue samples, or affects cognition.

[0011] It has been shown that one pathway for Aβ metabolism is via transport from CNS to the plasma (Zlokovic, B. V., *et al., Proc.* Natl. *Acad. Sci (USA)* (1996) 93:4229-4234; Ghersi-Egea, J-F., *et al., J. Neurochem.* (1996) 67:880-883). Additionally, it has been shown that Aβ in plasma can cross the blood-brain-barrier and enter the brain (Zlokovic, B. V., *et al., Biochem. Biophys. Res. Comm.* (1993) 67:1034-1040). It has also been shown that administration of certain polyclonal and monoclonal Aβ antibodies decreases Aβ deposition in amyloid plaques in the APP^{V717F} transgenic mouse model of Alzheimer's disease (Bard, F., *et al., Nature Med.* (2000) 6:916-919); however, this was said to be due to certain anti-Aβ antibodies crossing the blood-brain-barrier stimulating phagocytose of amyloid plaques by microglial cells. In Bard's experiments, assays of brain slices *ex vivo* showed that the presence of added Aβ antibody, along with exogenously added microglia, induced phagocytosis of Aβ, resulting in removal of Aβ deposits.

[0012] The levels of both soluble $A\beta_{40}$ and $A\beta_{42}$ in CSF and blood can readily be detected using standardized assays using antibodies directed against epitopes along the Aβ chain. Such assays have been reported, for example, in U.S. patents 5,766,846; 5,837,672; and 5,593,846. These patents describe the production of murine monoclonal antibodies to the central domain of the Aβ peptide, and these were reported to have epitopes around and including positions 16 and 17. Antibodies directed against the N-terminal region were described as well. Several monoclonal antibodies were asserted to immunoreact with positions 13-28 of the Aβ peptide; these did not bind to a peptide representing positions 17-28, thus, according to the cited patents, establishing that it is this region, including positions 16-17 (the α-secretase site) that was the target of these antibodies. Among antibodies known to bind between amino acids 13 and 28 of Aβ are mouse antibodies 266, 4G8, and 1C2.

[0013] We have now unexpectedly found that administration of the 266 antibody very quickly and almost completely restores cognition (object memory) in 24-month old hemizygous transgenic mice (APP^{V717F}). Yet, the antibody does not have the properties that the art teaches are required for an antibody to be effective in treating Alzheimer's disease, Down's syndrome, and other conditions related to the Aβ peptide. To our further surprise, we observed that antibodies that bind Aβ between positions 13 and 28 (266 and 4G8) are capable of sequestering soluble forms of Aβ from their bound, circulating forms in the blood, and that peripheral administration of antibody 266 results in rapid efflux of relatively large quantities of Aβ peptide from the CNS into the plasma. This results in altered clearance of soluble Aβ, prevention of plaque formation, and, most surprisingly, improvement in cognition, even without necessarily reducing Aβ amyloid plaque burden, crossing the blood brain barrier to any significant extent, decorating plaque, activating cellular mechanisms, or binding with great affinity to aggregated Aβ.

Disclosure of the Invention

[0014] The invention provides humanized antibodies, or fragments thereof, that positively affect cognition in diseases and conditions where Aβ may be involved, such as clinical or pre-clinical Alzheimer's disease, Down's syndrome, and clinical or pre-clinical cerebral amyloid angiopathy. The antibodies or fragments thereof need not cross the blood-brain barrier, decorate amyloid plaque, activate cellular responses, or even necessarily reduce amyloid plaque burden. In another aspect, this invention provides humanized antibodies and fragments thereof that sequester Aβ peptide from its bound, circulating form in blood, and alter clearance of soluble and bound forms of Aβ in central nervous system and plasma. In another aspect, this invention provides humanized antibodies and fragments thereof, wherein the humanized antibodies specifically bind to an epitope between amino acids 13 and 28 of the Aβ molecule. In another aspect, the invention provides humanized antibodies and fragments thereof, wherein the CDR are derived from mouse monoclonal antibody 266 and wherein the antibodies retain approximately the binding properties of the mouse antibody and have *in vitro* and *in vivo* properties functionally equivalent to the mouse antibody (sequences SEQ ID NO:1 through SEQ ID NO:6). In another aspect, this invention provides humanized antibodies and fragments thereof, wherein the variable regions have sequences comprising the CDR from mouse antibody 266 and specific human framework sequences (sequences SEQ ID NO:7 - SEQ ID NO:10), wherein the antibodies retain approximately the binding properties of the mouse antibody and have *in vitro* and *in vivo* properties functionally equivalent to the mouse antibody 266. In

another aspect, this invention provides humanized antibodies and fragments thereof, wherein the light chain is SEQ ID NO:11 and the heavy chain is SEQ ID NO:12.

[0015] Also part of the invention are polynucleotide sequences that encode the humanized antibodies or fragments thereof disclosed above, vectors comprising the polynucleotide sequences encoding the humanized antibodies or fragments thereof, host cells transformed with the vectors or incorporating the polynucleotides that express the humanized antibodies or fragments thereof, pharmaceutical formulations of the humanized antibodies and fragments thereof disclosed herein, and methods of making and using the same.

[0016] Such humanized antibodies and fragments thereof are useful for sequestering Aβ in humans; for treating and preventing diseases and conditions characterized by Aβ plaques or Aβ toxicity in the brain, such as Alzheimer's disease, Down's syndrome, and cerebral amyloid angiopathy in humans; for diagnosing these diseases in humans; and for determining whether a human subject will respond to treatment using human antibodies against Aβ.

[0017] Administration of an appropriate humanized antibody *in vivo* to sequester Aβ peptide circulating in biological fluids is useful for preventive and therapeutic treatment of conditions associated with the formation of Aβ-containing diffuse, neuritic, and cerebrovascular plaques in the brain. The humanized antibody, including an immunologically reactive fragment thereof, results in removal of the Aβ peptide from macromolecular complexes which would normally be relevant in transporting it in body fluids to and from sites where plaques can form or where it can be toxic. In addition, sequestering of plasma Aβ peptide with the antibody or fragment thereof behaves as a "sink," effectively sequestering soluble Aβ peptide in the plasma compartment, and inducing Aβ to enter the plasma from locations in the central nervous system (CNS). By sequestering Aβ in the blood, net efflux from the brain is enhanced and soluble Aβ is prevented from depositing in insoluble plaques and from forming toxic soluble species in the brain. In addition, insoluble Aβ in plaques which is in equilibrium with soluble Aβ can be removed from the brain through a sequestering effect in the blood. Sequestering the Aβ peptide with the antibody also enhances its removal from the body and inhibits toxic effects of soluble Aβ in the brain and the development and further accumulation of insoluble Aβ as amyloid in plaques. The antibodies useful in the invention do not cross the blood-brain barrier in large amounts (≤0.1% plasma levels). In addition, humanized antibodies used in the invention, when administered peripherally, do not need to elicit a cellular immune response in brain when bound to Aβ peptide or when freely circulating to have their beneficial effects. Further, when administered peripherally they do not need to appreciably bind aggregated Aβ peptide in the brain to have their beneficial effects.

[0018] Thus, in one aspect, the invention is directed to a method to treat and to prevent conditions characterized by the formation of plaques containing beta-amyloid protein in humans, which method comprises administering, preferably peripherally, to a human in need of such treatment a therapeutically or prophylactically effective amount of humanized monoclonal antibody or immunologically reactive fragment thereof, which antibody specifically binds to the mid-region of the Aβ peptide. In another aspect, the invention is directed to a method to inhibit the formation of amyloid plaques and to clear amyloid plaques in humans, which method comprises administering to a human subject in need of such inhibition an effective amount of a humanized antibody that sequesters Aβ peptide from its circulating form in blood and induces efflux out of the brain as well as altered Aβ clearance in plasma and the brain. In additional aspects, the invention is directed to such humanized antibodies, including immunologically effective portions thereof, and to methods for their preparation.

[0019] The invention also includes methods of reversing cognitive decline, improving cognition, treating cognitive decline, and preventing cognitive decline in a subject diagnosed with clinical or pre-clinical Alzheimer's disease, Down's syndrome, or clinical or pre-clinical cerebral amyloid angiopathy, comprising administering to the subject an effective amount of a humanized antibody of the invention.

[0020] The invention also includes use of a humanized antibody of the invention for the manufacture of a medicament, including prolonged expression of recombinant sequences of the antibody or antibody fragment in human tissues, for treating, preventing, or reversing Alzheimer's disease, Down's syndrome, or cerebral amyloid angiopathy; for treating, preventing, or reversing cognitive decline in clinical or pre-clinical Alzheimer's disease, Down's syndrome, or clinical or pre-clinical cerebral amyloid angiopathy; or to inhibit the formation of amyloid plaques or the effects of toxic soluble Aβ species in humans.

[0021] The invention is related to the surprising observation that within a short period of time after administration of an antibody of the present invention, relatively large quantities of Aβ efflux from the central nervous system to the blood. Thus, this invention includes methods to assess the response of a human subject to treatment with an antibody that binds Aβ or a fragment thereof, comprising: a) administering the antibody or a fragment thereof to the subject; and b) measuring the concentration of Aβ in the subject's blood.

[0022] The invention also includes a method of treating a human subject with an antibody that binds Aβ or a fragment thereof, comprising: a) administering a first amount of the antibody or fragment thereof to the subject; b) within 3 hours to two weeks after administering the first dose, measuring the concentration of Aβ in the subject's blood; c) if necessary, calculating a second amount of antibody or fragment thereof based on the result of step b), which second amount is the same as or different than the first amount; and d) administering the second amount of the antibody or fragment.

[0023] The invention also includes a method of assessing in a human subject the efficacy of an antibody that binds to Aβ, or a fragment thereof, for inhibiting or preventing Aβ amyloid plaque formation, for reducing Aβ amyloid plaque, for reducing the effects of toxic soluble Aβ species, or for treating a condition or a disease associated with Aβ plaque, comprising: a) obtaining a first sample of the subject's plasma or CSF; b) measuring a baseline concentration of Aβ in the first sample; c) administering the antibody or fragment thereof to the subject; d) within 3 hours to two weeks after administering the antibody or fragment thereof, obtaining a second sample of the subject's plasma or CSF; and e) measuring the concentration of Aβ in the second sample; wherein, efficacy is related to the quantity of Aβ bound to the antibody in the blood and the concentration of Aβ in the CSF.

Brief Description of the Drawings

[0024]

Figure 1 shows the percentage of the Aβ peptide withdrawn from human cerebrospinal fluid through a dialysis membrane by Mab 266 as a function of the molecular weight cutoff of the dialysis membrane.
Figure 2 shows the concentration of $A\beta_{Total}$ found in the plasma of an APP^{V717F} transgenic mouse after injection with either 200 µg or 600 µg of Mab 266 as a function of time.
Figure 3A shows the quantity of Aβ peptide deposition in the cortex in APP^{V717F} transgenic mice treated with saline, mouse IgG, or Mab 266. Figure 3B shows correlation of these results with parental origin.
Figure 4 shows the polynucleotide sequences for expressing humanized 266 light chain from plasmid pVk-Hu266 and the single amino acid codes for the expressed humanized 266 light chain (corresponding to SEQ ID NO:11 when mature).
Figure 5 shows the polynucleotide sequences for expressing humanized 266 heavy chain from plasmid pVgl-Hu266 and the single amino acid codes for the expressed humanized 266 heavy chain (corresponding to SEQ ID NO:12 when mature).
Figure 6 is a plasmid map of pVk-Hu266.
Figure 7 is a plasmid map of pVgl-Hu266.

Modes of Carrying Out the Invention

[0025] The Aβ peptides that circulate in human biological fluids represent the carboxy terminal region of a precursor protein encoded on chromosome 21. It has been reported from the results of *in vitro* experiments that the Aβ peptide has poor solubility in physiological solutions, since it contains a stretch of hydrophobic amino acids which are a part of the region that anchors its longer precursor to the lipid membranes of cells. It is thus not surprising that circulating Aβ peptide is normally complexed with other moieties that prevent it from aggregating. This has resulted in difficulties in detecting circulating Aβ peptide in biological fluids.

[0026] The above-mentioned patent documents (U.S. patents 5,766,846; 5,837,672 and 5,593,846) describe the preparation of antibodies, including a monoclonal antibody, designated clone 266 which was raised against, and has been shown to bind specifically to, a peptide comprising amino acids 13-28 of the Aβ peptide. The present applicants have found that antibodies that bind within this region, in contrast to antibodies that bind elsewhere in the amino acid sequence of Aβ, are able to sequester the soluble Aβ peptide very effectively from macromolecular complexes. This sequestration will effect net Aβ peptide efflux from the CNS, alter its clearance in CNS and plasma, and reduce its availability for plaque formation. Thus, antibodies of this specificity, modified to reduce their immunogenicity by converting them to a humanized form, offer the opportunity to treat, both prophylactically and therapeutically, conditions that are associated with formation ofbeta-amyloid plaques. These conditions include, as noted above, pre-clinical and clinical Alzheimer's, Down's syndrome, and pre-clinical and clinical cerebral amyloid angiopathy.

[0027] As used herein, the word "treat" includes therapeutic treatment, where a condition to be treated is already known to be present and prophylaxis - *i.e.*, prevention of, or amelioration of, the possible future onset of a condition.

[0028] By "monoclonal antibodies that bind to the mid-region of Aβ peptide" is meant monoclonal antibodies (Mab or Mabs) that bind an amino acid sequence representing an epitope contained between positions 13-28 of Aβ. The entire region need not be targeted. As long as the antibody binds at least an epitope within this region (especially, *e.g.*, including the α-secretase site 16-17 or the site at which antibody 266 binds), such antibodies are effective in the method of the invention.

[0029] By "antibody" is meant a monoclonal antibody *per se*, or an immunologically effective fragment thereof, such as an F_{ab}, $F_{ab'}$, or $F_{(ab')2}$ fragment thereof. In some contexts, herein, fragments will be mentioned specifically for emphasis; nevertheless, it will be understood that regardless of whether fragments are specified, the term "antibody" includes such fragments as well as single-chain forms. As long as the protein retains the ability specifically to bind its intended target, and in this case, to sequester Aβ peptide from its carrier proteins in blood, it is included within the term

"antibody." Also included within the definition "antibody" for example, are single chain forms, generally designated F_v regions, of antibodies with this specificity. Preferably, but not necessarily, the antibodies useful in the invention are produced recombinantly, as manipulation of the typically murine or other non-human antibodies with the appropriate specificity is required in order to convert them to humanized form. Antibodies may or may not be glycosylated, though glycosylated antibodies are preferred. Antibodies are properly cross-linked via disulfide bonds, as is well-known.

[0030] The basic antibody structural unit is known to comprise a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 kDa) and one "heavy" chain (about 50-70 kDa). The amino-terminal portion of each chain includes a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The carboxy-terminal portion of each chain defines a constant region primarily responsible for effector function.

[0031] Light chains are classified as gamma, mu, alpha, and lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, and define the antibody's isotype as IgG, IgM, IgA, IgD and IgE, respectively. Within light and heavy chains, the variable and constant regions are joined by a "J" region of about 12 or more amino acids, with the heavy chain also including a "D" region of about 10 more amino acids.

[0032] The variable regions of each light/heavy chain pair form the antibody binding site. Thus, an intact antibody has two binding sites. The chains all exhibit the same general structure of relatively conserved framework regions (FR) joined by three hypervariable regions, also called complementarily determining regions or CDRs. The CDRs from the two chains of each pair are aligned by the framework regions, enabling binding to a specific epitope. From N- terminal to C-terminal, both light and heavy chains comprise the domains FR1, CDR1, FR2, CDR2, FR3, CDR3 and FR4. The assignment of amino acids to each domain is in accordance with well known conventions [Kabat "Sequences of Proteins of Immunological Interest" National Institutes of Health, Bethesda, Md., 1987 and 1991; Chothia, et al., *J. Mol. Biol.* 196:901-917 (1987); Chothia, et al., *Nature* 342:878-883 (1989)].

[0033] As is well understood in the art, monoclonal antibodies can readily be generated with appropriate specificity by standard techniques of immunization of mammals, forming hybridomas from the antibody-producing cells of said mammals or otherwise immortalizing them, and culturing the hybridomas or immortalized cells to assess them for the appropriate specificity. In the present case such antibodies could be generated by immunizing a human, rabbit, rat or mouse, for example, with a peptide representing an epitope encompassing the 13-28 region of the Aβ peptide or an appropriate subregion thereof. Materials for recombinant manipulation can be obtained by retrieving the nucleotide sequences encoding the desired antibody from the hybridoma or other cell that produces it. These nucleotide sequences can then be manipulated to provide them in humanized form.

[0034] By "humanized antibody" is meant an antibody that is composed partially or fully of amino acid sequences derived from a human antibody germline by altering the sequence of an antibody having non-human complementarity determining regions (CDR). The simplest such alteration may consist simply of substituting the constant region of a human antibody for the murine constant region, thus resulting in a human/murine chimera which may have sufficiently low immunogenicity to be acceptable for pharmaceutical use. Preferably, however, the variable region of the antibody and even the CDR is also humanized by techniques that are by now well known in the art. The framework regions of the variable regions are substituted by the corresponding human framework regions leaving the non-human CDR substantially intact, or even replacing the CDR with sequences derived from a human genome. Fully human antibodies are produced in genetically modified mice whose immune systems have been altered to correspond to human immune systems. As mentioned above, it is sufficient for use in the methods of the invention, to employ an immunologically specific fragment of the antibody, including fragments representing single chain forms.

[0035] A humanized antibody again refers to an antibody comprising a human framework, at least one CDR from a non-human antibody, and in which any constant region present is substantially identical to a human immunoglobulin constant region, *i.e.*, at least about 85-90%, preferably at least 95% identical. Hence, all parts of a humanized antibody, except possibly the CDRs, are substantially identical to corresponding parts of one or more native human immunoglobulin sequences. For example, a humanized immunoglobulin would typically not encompass a chimeric mouse variable region/human constant region antibody.

[0036] Humanized antibodies have at least three potential advantages over non-human and chimeric antibodies for use in human therapy:

1) because the effector portion is human, it may interact better with the other parts of the human immune system (*e.g.*, destroy the target cells more efficiently by complement-dependent cytotoxicity (CDC) or antibody-dependent cellular cytotoxicity (ADCC)).

2) The human immune system should not recognize the framework or C region of the humanized antibody as foreign, and therefore the antibody response against such an injected antibody should be less than against a totally foreign non-human antibody or a partially foreign chimeric antibody.

3) Injected non-human antibodies have been reported to have a half-life in the human circulation much shorter than the half-life of human antibodies. Injected humanized antibodies will have a half-life essentially identical to

naturally occurring human antibodies, allowing smaller and less frequent doses to be given.

[0037] The design of humanized immunoglobulins may be carried out as follows. When an amino acid falls under the following category, the framework amino acid of a human immunoglobulin to be used (acceptor immunoglobulin) is replaced by a framework amino acid from a CDR-providing non-human immunoglobulin (donor immunoglobulin):

(a) the amino acid in the human framework region of the acceptor immunoglobulin is unusual for human immunoglobulin at that position, whereas the corresponding amino acid in the donor immunoglobulin is typical for human immunoglobulin at that position;
(b) the position of the amino acid is immediately adjacent to one of the CDRs; or
(c) any side chain atom of a framework amino acid is within about 5-6 angstroms (center-to-center) of any atom of a CDR amino acid in a three dimensional immunoglobulin model [Queen, *et al., op. cit.*, and Co, *et al., Proc. Natl. Acad. Sci. USA* 88, 2869 (1991)]. When each of the amino acid in the human framework region of the acceptor immunoglobulin and a corresponding amino acid in the donor immunoglobulin is unusual for human immunoglobulin at that position, such an amino acid is replaced by an amino acid typical for human immunoglobulin at that position.

[0038] A preferred humanized antibody is a humanized form of mouse antibody 266. The CDRs of humanized 266 have the following amino acid sequences:

light chain CDR1:

```
 1               5                  10                  15
Arg Ser Ser Gln Ser Leu Ile Tyr Ser Asp Gly Asn Ala Tyr Leu His
(SEQ ID NO:1)
```

light chain CDR2:

```
 1               5
Lys Val Ser Asn Arg Phe Ser  (SEQ ID NO:2)
```

light chain CDR3:

```
1               5
Ser Gln Ser Thr His Val Pro Trp Thr  (SEQ ID NO:3)
```

heavy chain CDR1:

```
 1               5
Arg Tyr Ser Met Ser (SEQ ID NO:4)
```

heavy chain CDR2

```
 1               5                  10                  15
Gln Ile Asn Ser Val Gly Asn Ser Thr Tyr Tyr Pro Asp Thr Val Lys Gly  (SEQ
ID NO:5)
```

and, heavy chain CDR3:

```
 1
Gly Asp Tyr  (SEQ ID NO:6).
```

[0039] A preferred light chain variable region of a humanized antibody of the present invention has the following amino acid sequence, in which the framework originated from human germline Vk segments DPK18 and J segment

Jk1, with several amino acid substitutions to the consensus amino acids in the same human V subgroup to reduce potential immunogenicity:

```
1               5                  10                  15
Asp Xaa Val Met Thr Gln Xaa Pro Leu Ser Leu Pro Val Xaa Xaa

                20                  25                  30
Gly Gln Pro Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu Xaa

                35                  40                  45
Tyr Ser Asp Gly Asn Ala Tyr Leu His Trp Phe Leu Gln Lys Pro

                50                  55                  60
Gly Gln Ser Pro Xaa Leu Leu Ile Tyr Lys Val Ser Asn Arg Phe

                65                  70                  75
Ser Gly Val Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp

                80                  85                  90
Phe Thr Leu Lys Ile Ser Arg Val Glu Ala Glu Asp Xaa Gly Val

                95                  100                 105
Tyr Tyr Cys Ser Gln Ser Thr His Val Pro Trp Thr Phe Gly Xaa

               110
Gly Thr Xaa Xaa Glu Ile Lys Arg                (SEQ ID NO:7)
```

wherein:

Xaa at position 2 is Val or Ile;
Xaa at position 7 is Ser or Thr;
Xaa at position 14 is Thr or Ser;
Xaa at position 15 is Leu or Pro;
Xaa at position 30 is Ile or Val;
Xaa at position 50 is Arg, Gln, or Lys;
Xaa at position 88 is Val or Leu;
Xaa at position 105 is Gln or Gly;
Xaa at position 108 is Lys or Arg; and
Xaa at position 109 is Val or Leu.

[0040] A preferred heavy chain variable region of a humanized antibody of the present invention has the following amino acid sequence, in which the framework originated from human germline VH segments DP53 and J segment JH4, with several amino acid substitutions to the consensus amino acids in the same human subgroup to reduce potential immunogenicity:

```
 1               5                  10                  15
Xaa Val Gln Leu Val Glu Xaa Gly Gly Gly Leu Val Gln Pro Gly

                20                  25                  30
Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser

                35                  40                  45
Arg Tyr Ser Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu

                50                  55                  60
Xaa Leu Val Ala Gln Ile Asn Ser Val Gly Asn Ser Thr Tyr Tyr

                65                  70                  75
Pro Asp Xaa Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Xaa

                80                  85                  90
Xaa Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Xaa Asp

                95                  100                 105
Thr Ala Val Tyr Tyr Cys Ala Ser Gly Asp Tyr Trp Gly Gln Gly

               110
Thr Xaa Val Thr Val Ser Ser                    (SEQ ID NO:8)
```

wherein:

Xaa at position 1 is Glu or Gln;
Xaa at position 7 is Ser or Leu;
Xaa at position 46 is Glu, Val, Asp, or Ser;
Xaa at position 63 is Thr or Ser;
Xaa at position 75 is Ala, Ser, Val, or Thr;
Xaa at position 76 is Lys or Arg;
Xaa at position 89 is Glu or Asp; and
Xaa at position 107 is Leu or Thr.

[0041] A particularly preferred light chain variable region of a humanized antibody of the present invention has the following amino acid sequence, in which the framework originated from human germline Vk segments DPK18 and J segment Jkl, with several amino acid substitutions to the consensus amino acids in the same human V subgroup to reduce potential immunogenicity:

```
1               5                  10                  15
Asp Val Val Met Thr Gln Ser Pro Leu Ser Leu Pro Val Thr Leu

            20                  25                  30
Gly Gln Pro Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu Ile

            35                  40                  45
Tyr Ser Asp Gly Asn Ala Tyr Leu His Trp Phe Leu Gln Lys Pro

            50                  55                  60
Gly Gln Ser Pro Arg Leu Leu Ile Tyr Lys Val Ser Asn Arg Phe

            65                  70                  75
Ser Gly Val Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp

            80                  85                  90
Phe Thr Leu Lys Ile Ser Arg Val Glu Ala Glu Asp Val Gly Val

            95                  100                 105
Tyr Tyr Cys Ser Gln Ser Thr His Val Pro Trp Thr Phe Gly Gln

           110
Gly Thr Lys Val Glu Ile Lys Arg                 (SEQ ID NO:9).
```

[0042] A particularly preferred heavy chain variable region of a humanized antibody of the present invention has the following amino acid sequence, in which the framework originated from human germline VH segments DP53 and J segment JH4:

```
 1              5                  10                  15
Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly

            20                  25                  30
Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser

            35                  40                  45
Arg Tyr Ser Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu

            50                  55                  60
Glu Leu Val Ala Gln Ile Asn Ser Val Gly Asn Ser Thr Tyr Tyr

            65                  70                  75
Pro Asp Thr Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala

            80                  85                  90
Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp

            95                  100                 105
Thr Ala Val Tyr Tyr Cys Ala Ser Gly Asp Tyr Trp Gly Gln Gly


           110
Thr Leu Val Thr Val Ser Ser                     (SEQ ID NO:10).
```

[0043] A preferred light chain for a humanized antibody of the present invention has the amino acid sequence:

```
 1               5                  10                  15
Asp Val Val Met Thr Gln Ser Pro Leu Ser Leu Pro Val Thr Leu

                20                  25                  30
Gly Gln Pro Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu Ile

                35                  40                  45
Tyr Ser Asp Gly Asn Ala Tyr Leu His Trp Phe Leu Gln Lys Pro

                50                  55                  60
Gly Gln Ser Pro Arg Leu Leu Ile Tyr Lys Val Ser Asn Arg Phe

                65                  70                  75
Ser Gly Val Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp

                80                  85                  90
Phe Thr Leu Lys Ile Ser Arg Val Glu Ala Glu Asp Val Gly Val

                95                 100                 105
Tyr Tyr Cys Ser Gln Ser Thr His Val Pro Trp Thr Phe Gly Gln

               110                 115                 120
Gly Thr Lys Val Glu Ile Lys Arg Thr Val Ala Ala Pro Ser Val

               125                 130                 135
Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly Thr Ala

               140                 145                 150
Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys

               155                 160                 165
Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln

               170                 175                 180
Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu

               185                 190                 195
Ser Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys

               200                 205                 210
Val Tyr Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val

               215
Thr Lys Ser Phe Asn Arg Gly Glu Cys              (SEQ ID NO:11}.
```

[0044] A preferred heavy chain for a humanized antibody of the present invention has the amino acid sequence:

```
 1               5                  10                  15
Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
```

```
                    20                  25                  30
Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser

                    35                  40                  45
Arg Tyr Ser Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu

                    50                  55                  60
Glu Leu Val Ala Gln Ile Asn Ser Val Gly Asn Ser Thr Tyr Tyr

                    65                  70                  75
Pro Asp Thr Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala

                    80                  85                  90
Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp

                    95                  100                 105
Thr Ala Val Tyr Tyr Cys Ala Ser Gly Asp Tyr Trp Gly Gln Gly

                    110                 115                 120
Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val

                    125                 130                 135
Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala

                    140                 145                 150
Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr

                    155                 160                 165
Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe

                    170                 175                 180
Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val

                    185                 190                 195
Val Thr Val Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys

                    200                 205                 210
Asn Val Asn His Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val

                    215                 220                 225
Glu Pro Lys Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro

                    230                 235                 240
Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro

                    245                 250                 255
Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr

                    260                 265                 270
Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe

                    275                 280                 285
Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys

                    290                 295                 300
Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val

                    305                 310                 315
Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys
```

```
                320                 325                 330
Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr

                335                 340                 345
Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr

                350                 355                 360
Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu

                365                 370                 375
Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu

                380                 385                 390
Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro

                395                 400                 405
Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu

                410                 415                 420
Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys

                425                 430                 435
Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser

                440
Leu Ser Leu Ser Pro Gly Lys                    (SEQ ID NO:12).
```

[0045] Other sequences are possible for the light and heavy chains for the humanized antibodies of the present invention and for humanized 266. The immunoglobulins can have two pairs of light chain/heavy chain complexes, at least one chain comprising one or more mouse complementarity determining regions functionally joined to human framework region segments.

[0046] In another aspect, the present invention is directed to recombinant polynucleotides encoding antibodies which, when expressed, comprise the heavy and light chain CDRs from an antibody of the present invention. As to the human framework region, a framework or variable region amino acid sequence of a CDR-providing non-human immunoglobulin is compared with corresponding sequences in a human immunoglobulin variable region sequence collection, and a sequence having a high percentage of identical amino acids is selected. Exemplary polynucleotides, which on expression code for the polypeptide chains comprising the heavy and light chain CDRs of monoclonal antibody 266 are given in Figures 4 and 5. Due to codon degeneracy and non-critical amino-acid substitutions, other polynucleotide sequences can be readily substituted for those sequences. Particularly preferred polynucleotides of the present invention encode antibodies, which when expressed, comprise the CDRs of SEQ ID NO:1 - SEQ ID NO:6, or any of the variable regions of SEQ ID NO:7 - SEQ ID NO:10, or the light and heavy chains of SEQ ID NO:11 and SEQ ID NO:12.

[0047] The polynucleotides will typically further include an expression control polynucleotide sequence operably linked to the humanized immunoglobulin encoding sequences, including naturally-associated or heterologous promoter regions. Preferably, the expression control sequences will be eukaryotic promoter systems in vectors capable of transforming or transfecting eukaryotic host cells, but control sequences for prokaryotic hosts may also be used. Once the vector has been incorporated into the appropriate host cell line, the host cell is propagated under conditions suitable for high level expression of the nucleotide sequences, and, as desired, the collection and purification of the light chains, heavy chains, light/heavy chain dimers or intact antibodies, binding fragments or other immunoglobulin forms may follow.

[0048] The nucleotide sequences of the present invention capable of ultimately expressing the desired humanized antibodies can be formed from a variety of different polynucleotides (genomic or cDNA, RNA, synthetic oligonucleotides, etc.) and components (*e.g.*, V, J, D, and C regions), as well as by a variety of different techniques. Joining appropriate genomic and synthetic sequences is a common method of production, but cDNA sequences may also be utilized.

[0049] Human constant region DNA sequences can be isolated in accordance with well known procedures from a variety of human cells, but preferably from immortalized B-cells. The CDRs for producing the immunoglobulins of the present invention will be similarly derived from non-human monoclonal antibodies capable of binding to an epitope between amino acids 13 and 28 of the Aβ peptide, which monoclonal antibodies are produced in any convenient mammalian source, including, mice, rats, rabbits, or other vertebrates capable of producing antibodies by well known methods, as described above. Suitable source cells for the polynucleotide sequences and host cells for immunoglobulin

expression and secretion can be obtained from a number of sources well-known in the art.

[0050] In addition to the humanized immunoglobulins specifically described herein, other "substantially homologous" modified immunoglobulins can be readily designed and manufactured utilizing various recombinant DNA techniques well known to those skilled in the art. For example, the framework regions can vary from the native sequences at the primary structure level by several amino acid substitutions, terminal and intermediate additions and deletions, and the like. Moreover, a variety of different human framework regions may be used singly or in combination as a basis for the humanized immunoglobulins of the present invention. In general, modifications of the genes may be readily accomplished by a variety of well-known techniques, such as site-directed mutagenesis.

[0051] Alternatively, polypeptide fragments comprising only a portion of the primary antibody structure may be produced, which fragments possess one or more immunoglobulin activities (e.g., complement fixation activity). These polypeptide fragments may be produced by proteolytic cleavage of intact antibodies by methods well known in the art, or by inserting stop codons at the desired locations in vectors using site-directed mutagenesis, such as after CH1 to produce Fab fragments or after the hinge region to produce $F(ab')_2$ fragments. Single chain antibodies may be produced by joining VL and VH with a DNA linker.

[0052] As stated previously, the encoding nucleotide sequences will be expressed in hosts after the sequences have been operably linked to (i.e., positioned to ensure the functioning of) an expression control sequence. These expression vectors are typically replicable in the host organisms either as episomes or as an integral part of the host chromosomal DNA. Commonly, expression vectors will contain selection markers, e.g., tetracycline or neomycin, to permit detection of those cells transformed with the desired DNA sequences. *E. coli* is a prokaryotic host useful particularly for cloning the polynucleotides of the present invention. Other microbial hosts suitable for use include bacilli, such as Bacillus subtilus, and other enterobacteriaceae, such as Salmonella, Serratia, and various Pseudomonas species. In these prokaryotic hosts, one can also make expression vectors, which will typically contain expression control sequences compatible with the host cell (*e.g.*, an origin of replication). In addition, any of a number of well-known promoters may be present, such as the lactose promoter system, a tryptophan (trp) promoter system, a beta-lactamase promoter system, or a promoter system from phage lambda. The promoters will typically control expression, optionally with an operator sequence, and have ribosome binding site sequences and the like, for initiating and completing transcription and translation.

[0053] Other microbes, such as yeast, may also be used for expression. Saccharomyces is a preferred host, with suitable vectors having expression control sequences, such as promoters, including 3-phosphoglycerate kinase or other glycolytic enzymes, and an origin of replication, termination sequences and the like as desired.

[0054] In addition to microorganisms, mammalian tissue cell culture may also be used to express and produce the polypeptides of the present invention. Eukaryotic cells are actually preferred, because a number of suitable host cell lines capable of secreting intact immunoglobulins have been developed in the art, and include the CHO cell lines, various COS cell lines, Syrian Hamster Ovary cell lines, HeLa cells, preferably myeloma cell lines, transformed B-cells, human embryonic kidney cell lines, or hybridomas. Expression vectors for these cells can include expression control sequences, such as an origin of replication, a promoter, an enhancer, and necessary processing information sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites, and transcriptional terminator sequences. Preferred expression control sequences are promoters derived from immunoglobulin genes, SV40, Adenovirus, Bovine Papilloma Virus, cytomegalovirus and the like.

[0055] The vectors containing the nucleotide sequences of interest (*e.g.*, the heavy and light chain encoding sequences and expression control sequences) can be transferred into the host cell by well-known methods, which vary depending on the type of cellular host. For example, calcium chloride transfection is commonly utilized for prokaryotic cells, whereas calcium phosphate treatment or electroporation may be used for other cellular hosts.

[0056] Once expressed, the whole antibodies, their dimers, individual light and heavy chains, or other immunoglobulin forms of the present invention can be purified according to standard procedures of the art, including ammonium sulfate precipitation, ion exchange, affinity, reverse phase, hydrophobic interaction column chromatography, gel electrophoresis and the like. Substantially pure immunoglobulins of at least about 90 to 95% homogeneity are preferred, and 98 to 99% or more homogeneity most preferred, for pharmaceutical uses. Once purified, partially or to homogeneity as desired, the polypeptides may then be used therapeutically or prophylactically, as directed herein.

[0057] The antibodies (including immunologically reactive fragments) are administered to a subject at risk for or exhibiting Aβ-related symptoms or pathology such as clinical or pre-clinical Alzheimer's disease, Down's syndrome, or clinical or pre-clinical amyloid angiopathy, using standard administration techniques, preferably peripherally *(i.e.* not by administration into the central nervous system) by intravenous, intraperitoneal, subcutaneous, pulmonary, transdermal, intramuscular, intranasal, buccal, sublingual, or suppository administration. Although the antibodies may be administered directly into the ventricular system, spinal fluid, or brain parenchyma, and techniques for addressing these locations are well known in the art, it is not necessary to utilize these more difficult procedures. The antibodies of the invention are effective when administered by the more simple techniques that rely on the peripheral circulation system. The advantages of the present invention include the ability of the antibody exert its beneficial effects even though not

provided directly to the central nervous system itself. Indeed, it has been demonstrated herein that the amount of antibody which crosses the blood-brain barrier is <0.1% of plasma levels and that the antibodies of the invention exert their ability to sequester Aβ in the peripheral circulation as well as to alter CNS and plasma soluble Aβ clearance.

[0058] The pharmaceutical compositions for administration are designed to be appropriate for the selected mode of administration, and pharmaceutically acceptable excipients such as dispersing agents, buffers, surfactants, preservatives, solubilizing agents, isotonicity agents, stabilizing agents and the like are used as appropriate. Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton PA, latest edition, incorporated herein by reference, provides a compendium of formulation techniques as are generally known to practitioners. It may be particularly useful to alter the solubility characteristics of the antibodies of the invention, making them more lipophilic, for example, by encapsulating them in liposomes or by blocking polar groups.

[0059] Peripheral systemic delivery by intravenous or intraperitoneal or subcutaneous injection is preferred. Suitable vehicles for such injections are straightforward. In addition, however, administration may also be effected through the mucosal membranes by means of nasal aerosols or suppositories. Suitable formulations for such modes of administration are well known and typically include surfactants that facilitate cross-membrane transfer. Such surfactants are often derived from steroids or are cationic lipids, such as N-[1-(2,3-dioleoyl)propyl-N,N,N-trimethylammoniumchloride (DOTMA) or various compounds such as cholesterol hemisuccinate, phosphatidyl glycerols and the like.

[0060] The concentration of the humanized antibody in formulations from as low as about 0.1% to as much as 15 or 20% by weight and will be selected primarily based on fluid volumes, viscosities, and so forth, in accordance with the particular mode of administration selected. Thus, a typical pharmaceutical composition for injection could be made up to contain 1 mL sterile buffered water of phosphate buffered saline and 1-100 mg of the humanized antibody of the present invention. The formulation could be sterile filtered after making the formulation, or otherwise made microbiologically acceptable. A typical composition for intravenous infusion could have a volume as much as 250 mL of fluid, such as sterile Ringer's solution, and 1-100 mg per mL, or more in antibody concentration. Therapeutic agents of the invention can be frozen or lyophilized for storage and reconstituted in a suitable sterile carrier prior to use. Lyophilization and reconstitution can lead to varying degrees of antibody activity loss (e.g. with conventional immune globulins, IgM antibodies tend to have greater activity loss than IgG antibodies). Dosages may have to be adjusted to compensate. The pH of the formulation will be selected to balance antibody stability (chemical and physical) and comfort to the patient when administered. Generally, pH between 4 and 8 is tolerated.

[0061] Although the foregoing methods appear the most convenient and most appropriate for administration of proteins such as humanized antibodies, by suitable adaptation, other techniques for administration, such as transdermal administration and oral administration may be employed provided proper formulation is designed.

[0062] In addition, it may be desirable to employ controlled release formulations using biodegradable films and matrices, or osmotic mini-pumps, or delivery systems based on dextran beads, alginate, or collagen.

[0063] In summary, formulations are available for administering the antibodies of the invention and are well-known in the art and may be chosen from a variety of options.

[0064] Typical dosage levels can be optimized using standard clinical techniques and will be dependent on the mode of administration and the condition of the patient.

[0065] The following examples are intended to illustrate but not to limit the invention.

[0066] The examples hereinbelow employ, among others, a murine monoclonal antibody designated "266" which was originally prepared by immunization with a peptide composed of residues 13-28 of human Aβ peptide. The antibody was confirmed to immunoreact with this peptide, but had previously been reported to not react with the peptide containing only residues 17-28 of human Aβ peptide, or at any other epitopes within the Aβ peptide. The preparation of this antibody is described in U.S. patent 5,766,846, incorporated herein by reference. As the examples here describe experiments conducted in murine systems, the use of murine monoclonal antibodies is satisfactory. However, in the treatment methods of the invention intended for human use, humanized forms of the antibodies with the immunospecificity corresponding to that of antibody 266 are preferred.

Example 1

Sequestration of Added Aβ Peptide in Human Fluids

[0067] Samples of human cerebrospinal fluid (CSF) (50 µl) and human plasma (50 µl) were incubated for 1 hour at room temperature as follows:

1. alone;
2. along with 5 ng Aβ 40 peptide; or
3. 5 ng Aβ 40 peptide plus 1 mg monoclonal antibody 266 (described, for example, in U.S. patent 5,766,846 incorporated herein by reference).

[0068] The samples were then electrophoresed on a 4-25% non-denaturing gradient gel, *i. e.*, non-denaturing gradient electrophoresis (NDGGE) and transferred to nitrocellulose. The blots were then stained with Ponceau S or, for Western blot, probed with biotin-labeled monoclonal antibody (3D6) which is directed against the first five amino acids of Aβ peptide, developed with streptavidin-horse radish peroxidase and detected by enhanced chemiluminescence (ECL). The hydrated diameters of the materials contained in bands on the blots were estimated using Pharmacia molecular weight markers. Thus, if the Aβ peptide is bound to other molecules, it would run at the size of the resulting complex.

[0069] Western blots of CSF either with or without 5 ng Aβ peptide shows no evidence of the Aβ peptide in response to detection mediated by antibody 3D6. Similar results are obtained for human plasma. This was true despite the fact that Aβ peptide could be detected by SDS-PAGE followed by Western blot using the same technique and on the same CSF samples. Presumably, the detection of Aβ peptide was prevented by interactions between this peptide and other factors in the fluids tested. However, when Mab 266 is added to the incubation, characteristic bands representing sequestered Aβ peptide complexed to the antibody are present both in plasma and in CSF. The major band is at approximately 11 nm hydrated diameter, corresponding to antibody monomer with an additional smaller band at 13 nm corresponding to antibody dimer.

Example 2

Specificity of the Sequestering Antibody

[0070] Samples containing 50 µl of human CSF or 10 µl of APP^{V717F} CSF were used. APP^{V717F} are transgenic mice representing a mouse model of Alzheimer's disease in which the human amyloid precursor protein transgene with a familial Alzheimer's disease mutation is expressed and results in the production of human Aβ peptide in the central nervous system.

[0071] The samples were incubated with or without various Mabs (1 µg) for 1 hour at room temperature and then electrophoresed on a 4-25% NDGGE and blotted onto nitrocellulose as described in Example 1. The antibodies were as follows:

Mab 266 (binds to positions 13-28);
Mab 4G8 (binds to positions 17-24);
QCBpan (rabbit polyclonal for positions 1-40);
mouse IgG (non-specific);
Mab 3D6 (binds to positions 1-5);
Mab 21F12 (binds to positions 33-42):
Mab 6E10 (binds to positions 1-17); and
$QCB_{40, 42}$ (rabbit polyclonals to $A\beta_{40}$ and $A\beta_{42}$).

[0072] Detection of the Aβ peptide antibody complex was as described in Example 1 - biotin labeled 3D6 (to the Aβ peptide N-terminus) followed by streptavidin-HRP and ECL. Similar detection in human CSF incubated with Mab 266, in some instances substituted $QCB_{40, 42}$, which binds to the carboxyl terminus of Aβ peptide, for 3D6.

[0073] The results showed that of the antibodies tested, only Mab 4G8 and Mab 266 permitted the detection of Aβ peptide.

[0074] The results showed that for human CSF, only Mab 266 and Mab 4G8 were able to sequester in detectable amounts of an antibody Aβ complex (again, without any antibody, no Aβ is detected). Mab 266 was also able to produce similar results to those obtained with human CSF with CSF from APP^{V717F} transgenic mice. Aβ peptide could be sequestered in human CSF using Mab 266 regardless of whether 3D6 or $QCB_{40,42}$ antibody was used to develop the Western blot.

Example 3

Demonstration of Aβ Peptide -266 Complex by Two-Dimensional Electrophoresis

[0075] A sample containing 50 ng $A\beta_{40}$ peptide was incubated with 2 µg Mab 266 at 37°C for 3 hours. A corresponding incubation of Mab 266 alone was used as a control.

[0076] The samples were then subjected to 2-dimensional gel electrophoresis.

[0077] In the first dimension, the incubated samples were subjected to NDGGE as described in Example 1. The polyacrylamide gel was then cut into individual lanes perpendicular to the direction of the first dimensional flow and gel separation under denaturing/reducing conditions by SDS-PAGE (Tricine urea gel) was performed in the second

dimension. The presence of the bands was detected either by Ponceau-S staining (any protein) or by specific development using 6E10 Mab (Senetek, Inc.) and biotinylated anti-mouse Aβ in the HRP-based detection system.

[0078] Ponceau-S staining of the nitrocellulose blots after transfer permitted visualization of the heavy and light chains of Mab 266 alone. It was confirmed that Aβ peptide was in a complex with Mab 266 as a band at 4 kD was observed that aligns with the size of full-length Mab 266 seen after the first dimension NDGGE.

Example 4

Demonstration of Non-Equivalence of Binding and Sequestration

[0079] Aβ peptide as it circulates in plasma and CSF is thought to be contained in a complex with proteins, including apolipoprotein E. The present example demonstrates that antibodies to apoE, while able to bind to the complex, do not sequester apoE from the remainder of the complex.

[0080] ApoE complexes (500 ng) were incubated with Mab or polyclonal antibodies to apoE (2 µg) at 37°C for one hour. The incubated samples were then subjected to NDGGE using the techniques described in Example 1. Following NDGGE, Western blotting was performed with affinity purified goat anti-apoE antibodies with detection by ECL. When no antibody is present, apoE can be detected at 8-13 nm consistent with its presence in lipoprotein particles. The presence of monoclonal or polyclonal antibodies to apoE results in a population shift of apoE to a larger molecular species, a "super shift". This demonstrates that the antibodies to apoE did not sequester, *i.e.*, remove apoE from a lipoprotein particle, rather they bind to apoE on the lipoproteins creating a larger molecular species.

Example 5

Sequestration of Aβ is Not Perturbed by Anti-apoE Antibodies

[0081] A sample of 100 µl human CSF was incubated either with Mab 266 alone, or with polyclonal anti-apoE, or with both antibodies for 60 minutes at 37°C. The samples were then analyzed by NDGGE as described in Example 1 and the detection of bands performed as described in Example 1.

[0082] The results show that as long as Mab 266 was added to the sample, the band at approximately 11 nm diameter characteristic of the sequestered 266-Aβ peptide complex was visible. This is the case whether or not anti-apoE is present. This band, demonstrating sequestered Aβ, also appears if 50 ng of Aβ peptide is added to the incubation mixture in the presence of Mab 266. Thus, alteration of the molecular weight of apoE by the presence of anti-apoE antibodies does not interfere with sequestration of Aβ peptide by Mab 266.

Example 6

Sequestration of Aβ Peptide *In Vivo*

[0083]

A. Transgenic APP^{V717F} mice, also termed PDAPP mice, over-express a mutant form of human APP protein. These mice produce human Aβ in the CNS and have elevated levels of human Aβ peptide circulating in the CSF and plasma. Eight month old mice were injected intravenously with saline or 100 µg of Mab 266. They were bled 10 minutes after initial injection and again at 20 hours after initial injection.

Samples containing 20 µl of plasma from each animal were analyzed by NDGGE and Western blot with antibody 3D6 as described in Example 1. The saline injected animals did not show the presence of the characteristic 11 nm sequestered Aβ peptide band either after 10 minutes or 20 hours. However, the two animals that were injected with Mab 266 did show the appearance of this band after 20 hours.

B. Two month old APP^{V717F} mice were used in this study. At day zero, the mice received either no Mab 266, 1 mg Mab 266, or 100 µg of this antibody. Plasma samples were taken two days prior to administration of the antibodies and on days 1, 3, 5 and 7. The plasma samples were subjected to NDGGE followed by Western blotting and detection with 3D6 as described in Example 1. At all time points following administration of Mab 266, the 266/Aβ complex was detected unless the plasma sample had been treated with protein G, which binds to immunoglobulin, thus effectively removing the Mab 266. Consistent levels of complex over the time period tested were found except for a slight drop-off at day seven in animals injected with 100 µg of Mab 266; in general, the levels in animals administered 100 µg were consistently lower than those found in the mice administered 1 mg of this antibody.

C. Two two-month old APP^{V717F} mice were administered 1 mg of Mab 266 intravenously and a 25 µl plasma sample was taken from each. The plasma sample was subj ected to NDGGE followed by Western blot as described above

except that binding with biotinylated 3D6 was followed by detection with streptavidin^{125}I(Amersham) and exposure to a phosphorimaging screen. The level of complex was estimated in comparison to a standard curve using known amounts of $A\beta_{40}$ complexed with saturating levels of Mab 266 and detected similarly. The amount of Aβ peptide bound to Mab 266 was estimated at approximately 100 ng/ml, representing an increase of approximately 1,000-fold over endogenous Aβ peptide in these mice which had been determined to be about 100 pg/ml. This is also similar to the level of Aβ peptide in APP^{V717F} brain prior to Aβ deposition (50-100 ng/g); human APP and human Aβ in APP^{V717F} Tg mice are produced almost solely in the brain. Thus, it appears that the presence of Mab 266 in the plasma acts as an Aβ peptide sink facilitating net efflux of Aβ peptide from the CNS into the plasma. This increased net efflux likely results from both increasing Aβ efflux from CNS to plasma and also from preventing Aβ in plasma from re-entering the brain.

The correct size for the sequestered Aβ peptide was confirmed by running 20 µL of plasma samples obtained from APP^{V717F} mice 24 hours after being injected with 1 mg Mab 266 on TRIS-tricine SDS-PAGE gels followed by Western blotting using anti-Aβ antibody 6E10 prior to, or after, protein G exposure using protein G-bound beads. A band that was depleted by protein G was detected at 4-8 kD, consistent with the presence of monomers and possibly dimers of Aβ peptide.

D. Two month old APP^{V717F} mice were treated with either PBS (n=7) or 500 µg biotinylated Mab 266 - *i.e.*, m266B (n=9) intraperitoneally. Both prior to and 24 hours after the injection, plasma was analyzed for total Aβ peptide using a modification of the ELISA method of Johnson-Wood, K., *et al., Proc. Natl. Acad. Sci. USA* (1997) 94: 1550-1555; and Bales, K.R, *et al., Nature Genet* (1997) 17:263-264. Total Aβ bound to m266B was measured by using 96-well Optiplates (Packard, Inc.) coated with m3D6. Diluted plasma samples and standards (varying concentrations of $A\beta_{40}$ and m266B) were incubated overnight in the coated plates and the amount of total Aβ/m266B complex was determined with the use of ^{125}I-Streptavidin. In addition, at the 24-hour time point, the plasma samples were first treated with protein G to quantitate Aβ peptide not bound to Mab 266, and $A\beta_{Total}$ and $A\beta_{42}$ were determined by ELISA in the CSF. In PBS-injected animals, plasma Aβ peptide levels were 140 pg/ml both before and after injection. Plasma levels were similar in the Mab 266-injected mice prior to injection, but levels of Aβ peptide not bound to Mab 266 were undetectable at 24 hours post injection.

[0084] Levels in the CSF were also measured, CSF represents an extracellular compartment within the CNS and concentration of molecules in the CSF reflects to some extent the concentration of substances in the extracellular space of the brain. CSF was isolated from the cisterna magna compartment. Mice were anesthetized with pentobarbital and the musculature from the base of the skull to the first vertebrae was removed. CSF was collected by carefully puncturing the arachnoid membrane covering the cistern with a micro needle under a dissecting microscope and withdrawing the CSF into a polypropylene micropipette. At 24 hours post injection, an increase in total Aβ peptide in the CSF of Mab 266-injected mice was found, and an approximately two-fold increase in $A\beta_{42}$ as compared to PBS injected mice was obtained in the CSF. This was confirmed using denaturing gel electrophoresis followed by Western blotting with $A\beta_{42}$-specific antibody 21F12.

[0085] In an additional experiment, three month old APP^{V717F} Tg mice were injected with either PBS or Mab 266 intravenously and both $A\beta_{40}$ and $A\beta_{42}$ levels were assessed in the CSF as follows:

[0086] For measurement of $A\beta_{40}$, the monoclonal antibody m2G3, specific for $A\beta_{40}$ was utilized. The ELISA described (Johnson-Wood, K., *et al., Proc. Natl. Acad. Sci. USA* (1997) 94:1550-1555) was modified into an RIA by replacing the Streptavidin-HRP reagent with ^{125}I-Streptavidin. For plasma and CSF samples, the procedure was performed under non-denaturing conditions that lacked guanidine in the buffers. For assessment of carbonate soluble and insoluble Aβ in brain homogenate, samples were homogenized with 100 mM carbonate, 40 mM NaCl, pH 11.5 (4°C), spun at 10,000 x g for 15 min, and Aβ was assessed in the supernatant (soluble) and the pellet (insoluble) fractions as described (Johnson-Wood, K., *et al., Proc. Natl. Acad. Sci. USA* (1997) 94:1550-1555) and listed above. The measurement of Aβ/Mab 266 complex in plasma was performed by a modified RIA. Mice were injected with biotinylated Mab 266 (Mab 266B) and plasma was isolated at multiple time points. Total Aβ bound to Mab 266 was measured by using 96-well Optiplates (Packard, Inc.) coated with m3D6. Diluted plasma samples and standards (varying concentrations of $A\beta_{40}$ and Mab 266B) were incubated overnight in the coated plates and the amount of total Aβ/Mab 266B complex was determined with the use of ^{125}I-Streptavidin.

[0087] Three hours following the intravenous injection of Mab 266, there was a two-fold increase in CSF $A\beta_{40}$ levels and a non-significant increase in $A\beta_{42}$. However, at both 24 and 72 hours there was a two to three-fold increase in both $A\beta_{40}$ and $A\beta_{42}$ in the CSF. Similar results were obtained using denaturing gel analysis followed by Aβ Western blotting of pooled CSF. The efflux of Aβ through brain interstitial fluid, which is reflected to some degree by CSF levels, likely accounts for the observed increase in CSF Aβ.

[0088] It is significant that the change in CSF Aβ peptide levels cannot be due to entry of Mab 266 into the CSF since the levels measured 24 hours after injection, which are less than 0.1% plasma levels of Mab 266, are insufficient to account for the changes. These results suggest Aβ peptide is withdrawn from the brain parenchyma into the CSF by

the presence of the antibody in the bloodstream.

[0089] Forms of Aβ peptide which are soluble in PBS or carbonate buffer were measured in cerebral cortical homogenates in the same mice which had been injected with Mab 266 and in which the CSF was analyzed as described above. Similar increases in these soluble forms in the cortical homogenates were observed.

Example 7

Mab 266 Acts as an Aβ Peptide Sink *In Vitro*

[0090] A dialysis chamber was constructed as an *in vitro* system to test the ability of Mab 266 to act as a sink for Aβ peptide. One mL of human CSF was placed in the top chamber of a polypropylene tube separated by a dialysis membrane with a specified cutoff in the range 10-100 kD from a bottom chamber containing 75 µL PBS with or without 1 µg of Mab 266.

[0091] It appeared that equilibrium was reached after 3 hours, as determined by subjecting material in the bottom chamber to acid urea gels followed by Western blotting for Aβ peptide with 6E10 at various time points. Samples were denatured in formic acid to a final concentration of 80% (vol/vol) and reduced with β-mercaptoethanol (1%). Samples were electrophoresed (anode to cathode) in a 0.9 M acetic acid running buffer through a 4% to 35% polyacrylamide gradient gel containing 6 M urea, 5% (vol/vol) glacial acetic acid, and 2.5% TEMED. The acidic pH of the gel was neutralized prior to transfer to nitrocellulose. Subsequently, standard Western blotting techniques were used to identify Aβ. The bands detected correspond to 4 kD.

[0092] The amount of Aβ removed from the top chamber was thus determined by ELISA analysis of both top and bottom chambers (n=4) after 3 hours. The results for various molecular weight cutoffs in the presence and absence of Mab 266 are shown in Figure 1. As shown, while only minimal amounts of Aβ peptide crossed the membrane when PBS was placed in the bottom chamber, 50% of the Aβ peptide was sequestered in the bottom chamber when Mab 266 was present and the molecular weight cutoff was 25 kD; increasing amounts crossed as the molecular weight cutoff increased to 100 kD, when almost 100% of the Aβ peptide was drawn across the membrane.

[0093] It was also observed that the anti-N-terminal Aβ antibodies 3D6 and 10D5 were able to draw Aβ peptide across the membrane in this system, though not able to sequester Aβ peptide in the assays described in Example 1. These results show that antibodies to the Aβ peptide have sufficient affinity under these conditions to sequester the peptide in physiological solutions away from other binding proteins, but that Mabs such as 266 which are immunoreactive with an epitope in positions 13-28 are substantially more efficient and bind with higher affinity.

[0094] In similar assays, astrocyte-secreted apoE4 which was purified as described by DeMattos, R.B., *et al., J. Biol. Chem* (1998) 273:4206-4212; Sun, Y., *et al., J. Neurosci.* (1998) 18:3261-3272, had a small by statistically significant effect in increasing the mass of Aβ peptide in the bottom chamber. No apparent affect was observed when polyclonal IgG or BSA was substituted for Mab 266.

Example 8

Flux of Aβ Peptide into Plasma from the CNS

[0095]

A. One µg of $A\beta_{40}$ was dissolved into 5 µL of rat CSF to keep it soluble and was then injected into the subarachnoid space of the cisterna magna of wild-type Swiss-Webster mice which had previously received IV injections of either PBS (n=3) or 200 µg of biotinylated Mab 266 (n=3). At different time-points following treatment, $A\beta_{Total}$ in the plasma of the mice was determined by Aβ ELISA, using 3D6 as the coating antibody and standards of Aβ mixed with an excess of biotinylated 266. Each plasma sample was spiked with an excess of biotinylated 266 after removal from each animal for Aβ detection in the ELISA. In the PBS-injected mice, minimally detectable amounts of the peptide at levels of 0.15 ng/ml were detected as peak values after 30-60 minutes, after which the levels were essentially zero. In the mice administered Mab 266, however, plasma Aβ peptide reached levels 330-fold higher than those detected in PBS-injected mice after 60 minutes (approximately 50 ng/ml) and reached values of approximately 90 ng/ml after 180 minutes.

B. This procedure was repeated using either 200 µg (n=3) or 600 µg (n=3) injected IV into two-month-old APP^{V717F} mice. Mab 266 was injected i.v. into 3 month old APP^{V717F} +/+ mice with the above doses. Prior to and at different time-points following i.v. injection, the plasma concentration of Aβ bound to Mab 266 was determined by RIA. The detailed results from one illustrative mouse are shown in Figure 2.

[0096] It was found that the concentration of Aβ bound to the monoclonal antibody Mab 266 increased from basal

levels of 150 pg/ml to levels of over 100 ng/ml by four days. By analyzing early time points on the curve, it was determined that the net rate of entry of $A\beta_{Total}$ into plasma of the APP^{V717F} Tg mice was 42 pg/ml/minute in the presence of saturating levels of the antibody.

[0097] The effects of Mab 266 on plasma Aβ levels in both wild type and APP^{V717F} Tg mice as well as the effects of the antibody on Aβ concentration in CSF show that the presence of circulating Mab 266 results in a change in the equilibrium of Aβ flux or transport between the CNS and plasma.

Example 9

Mab 266 Effect on Aβ in the Brain

[0098] Four month old $APP^{V717F+/+}$ mice were treated every 2 weeks for 5 months with IP injections of saline, Mab 266 (500 μg), or control mouse IgG (100 μg, Pharmigen). The mice were sacrificed at nine months of age, and Aβ deposition in the cortex was determined. The % area covered by Aβ-immunoreactivity, as identified with a rabbit pan-Aβ antibody (QCB, Inc.), was quantified in the cortex immediately overlying the dorsal hippocampus as described by Holtzman, D.M., *et al., Ann. Neurol.* (2000) 97:2892-2897. The results are shown in Figure 3A. At this age, about half of each group has still not begun to develop Aβ deposition. However, the % of mice with >50% Aβ burden in the cortex was significantly less (P=0.02, Chi-square test) in the 266-treated group. While APP^{V717F} mice can develop large amounts of Aβ deposits by nine months, there is great variability with about 50% showing no deposits and about 50% showing substantial deposits. In PBS and IgG treated animals, 6/14 and 5/13 mice had greater than 50% of the cortex covered by Aβ staining, while only one of 14 mice treated with Mab 266 had this level of staining. Almost 50% of the animals in all groups still had not developed Aβ deposition by 9 months of age. The latter appears to be due to parental origin of individual mice in our cohort since even though all mice studied were confirmed to be $APP^{V717F+/+}$, high levels of Aβ deposition was observed only in mice derived from 4/8 breeding pairs (High pathology litters). Mice derived from the other 4 breeding pairs were virtually free of Aβ deposits (Low pathology litters). Using parental origin as a co-variate, there was a strong, significant effect of m266 in reducing Aβ deposition (p=0.0082, Fig. 3B).

Example 10

Peripherally injected Mab 266 does not bind to plaques in APPV717F Tg mice

[0099] To determine whether Mab 266 injected i.p. over 5 months was bound to Aβ in brain, brain sections from 9 month old $APP^{V717F+/+}$ Tg mice which contained Aβ deposits and had been treated with either Mab 266, saline, or control IgG were utilized. Tissue processing and immunostaining was performed as described (Bales, K.R., *et al., Nature Genet.* (1997) 17:263-264). Tissue from all groups of animals was incubated with fluorescein-labeled anti-mouse IgG (Vector, Inc.) and then examined under a fluorescent microscope. No specific staining of Aβ deposits was seen in any of the groups. In contrast, when applying Mab 266 to sections prior to incubation of the sections with anti-mouse IgG, Aβ deposits were clearly detected.

Example 11

Effect of administration of antibody 266 on cognition in 24-month old transgenic, hemizygous PDAPP mice

[0100] Sixteen hemizygous transgenic mice (APP^{V717F}) were used. The mice were approximately 24 months old at the start of the study. All injections were intraperitoncal (i.p.). Half the mice received weekly injections of phosphate buffered saline (PBS, "Control") and the other half received 500 micrograms of mouse antibody 266 dissolved in PBS. Injections were made over a period of seven weeks (42 days) for a total of six injections. Three days following the last injection, the behavior of the animals was assessed using an object recognition task, essentially as described in J.-C. Dodart, *et al., Behavioral Neuroscience,* 113 (5) 982-990 (1999). A recognition index $(T_B \times 100)/(T_B-T_A)$ was calculated. Results are shown below in Table 1.

Table 1. Descriptive statistics for recognition index

		Recognition Index (minutes)		
	N	Mean	Standard Deviation	Standard Error
Control (PBS)	8	71.2**	8.80	3.11
Antibody 266	8	54.35	7.43	2.62

** p=0.0010

[0101] Administration of 500 micrograms of antibody 266 weekly to 24 month old, hemizygous, transgenic mice was associated with a significant change in behavior. Antibody treated transgenic mice had recognition indices which were similar to wildtype control animals [J.-C. Dodart, *et al*]. The difference in the recognition index was statistically significant at the 0.001 probability level. The increased recognition index is an indication that treatment with an antibody that binds to the beta amyloid peptide in the region of amino acids 13-28 will reverse the behavioral impairments that had been documented in this mouse model of Alzheimer's Disease. Therefore, the administration of antibodies that bind beta amyloid peptide in the region of amino acids 13-28 will treat diseases such as Alzheimer's disease and Down's syndrome and will halt the cognitive decline typically associated with disease progression.

[0102] The amyloid burden (% area covered by immunoreactive material after staining with anti-Aβ antibodies 3D6 or 21F12) was quantified in the cortex immediately overlying the hippocampus including areas of the cingulate and parietal cortex from the brains of the 24 month-old animals treated with mouse antibody 266 for seven weeks, as described above. The results are presented in the table below. The differences between the treatment groups are not statistically significant.

Table 2. Amyloid plaque burden in APP $^{V717F+/-}$ mice following treatment with mouse 266 anti-Aβ antibody

		Plaque Burden (%)			
		Using 3D6		Using 21F12	
	N	Mean	Standard Error	Mean	Standard Error
Control (PBS)	7	44.3	5.93	0.77	0.14
Antibody 266	8	38.0	2.96	0.93	0.11

[0103] For these very old animals, treatment with mouse antibody 266 did not result in a significantly different amyloid burden compared with the PBS-treated group, measured using either 3D6 or using 21F12. Furthermore, the Aβ burden was substantially greater and significantly increased compared with the amyloid burden in younger animals (see below) who were not able to discriminate a novel object from a familiar one in the object recognition task. Most surprisingly, these results demonstrate that anti-Aβ antibodies can reverse cognitive deficits without the need to reduce amyloid burden *per se*.

[0104] After 7 weeks of treatment, the recognition index of the m266-treated group was not significantly different than what would be expected for a wild type cohort of 24 month old mice! This indicates a complete reversal of cognitive decline in these transgenic animals.

Example 12

Effect of administration of antibody 266 on cognition in young transgenic, hemizygous PDAPP mice

[0105] Fifty-four (54) homozygous, transgenic mice (APP^{V717F}) were used. Twenty-three (23) mice were approximately two months old at the start of the study. The remaining mice were approximately four months old at the start of the study. The duration of treatment was five months. Thus, at study termination, the mice were either approximately

seven (7) months old or approximately nine (9) months old.

[0106] All injections were intraperitoneal (i.p.). Each mouse in "PBS" control groups received a weekly injection of phosphate buffered saline (PBS; 200 µL). Each mouse in the "IgG" control groups received a weekly injection of IgG1κ1 isotype control (100 µg/mouse/week). Each mouse in the "High Dose" groups received a weekly injection of 500 microgram of antibody 266 dissolved in PBS ("HD"). Each mouse in the "Low Dose" group received a weekly injection of 100 micrograms of antibody 266 dissolved in PBS ("LD"). Three days following the last injection, the behavior of the animals was assessed using an object recognition task, as described in Example 10 above, and a discrimination index was calculated as the difference between the time spent on a novel object and the time spent on a familiar object. Results are shown below in Table 3. The data are grouped by the age of the mice at the end of the study.

Table 3. Descriptive statistics for discrimination index

		Discrimination Index (minutes)		
	N	Mean	Standard Deviation	Standard Error
7 months old				
PBS	7	2.12	4.22	1.59
IgG	8	0.81	3.64	1.29
HD	8	10.04*	6.52	2.30
9 months old				
PBS	7	1.87	3.54	1.34
IgG	8	0.96	3.51	1.24
LD	8	10.75*	6.44	2.28
HD	8	12.06***	7.82	2.76

*$p<0.05$

***$p<0.0001$

[0107] Taken together these data support the conclusion that administration of antibody 266, an antibody directed against the central domain of Aβ, attenuates plaque deposition in 7-9 month old APP^{V717F} transgenic mice, as well as reverses the behavioral impairments previously characterized. Treatment of patients with an antibody directed against the central domain of the Aβ peptide will inhibit or prevent cognitive decline typically associated with disease progression, and will reverse it.

[0108] The discrimination index for treated animals was not significantly different than what would be expected for wild type mice of the same age. Thus, just as in older animals (Example 11), treatment with m266 completely reversed cognitive decline in these younger transgenic animals.

Example 13

Synthesis of Humanized Antibody 266

[0109] Cells and antibodies. Mouse myeloma cell line Sp2/0 was obtained from ATCC (Manassas, VA) and maintained in DME medium containing 10% FBS (Cat # SH32661.03, HyClone, Logan, UT) in a 37°C CO_2 incubator. Mouse 266 hybridoma cells were first grown in RPMI-1640 medium containing 10% FBS (HyClone), 10 mM HEPES, 2 mM glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 25 µg/ml gentamicin, and then expanded in serum-free media (Hybridoma SFM, Cat # 12045-076, Life Technologies, Rockville, MD) containing 2% low Ig FBS (Cat # 30151.03, HyClone) to a 2.5 liter volume in roller bottles. Mouse monoclonal antibody 266 (Mu266) was purified from the culture supernatant by affinity chromatography using a protein-G Sepharose column. Biotinylated Mu266 was prepared using EZ-Link Sulfo-NHS-LC-LC-Biotin (Cat # 21338ZZ, Pierce, Rockford, IL).

[0110] Cloning of variable region cDNAs. Total RNA was extracted from approximately 10^7 hybridoma cells using TRIzol reagent (Life Technologies) and poly(A)$^+$ RNA was isolated with the PolyATract mRNA Isolation System (Promega, Madison, WI) according to the suppliers' protocols. Double-stranded cDNA was synthesized using the SMART™-RACE cDNA Amplification Kit (Clontech, Palo Alto, CA) following the supplier's protocol. The variable region cDNAs for the light and heavy chains were amplified by polymerase chain reaction (PCR) using 3' primers that anneal respectively to the mouse kappa and gamma chain constant regions, and a 5' universal primer provided in the SMART™RACE cDNA Amplification Kit. For VL PCR, the 3' primer has the sequence:

```
5'-TATAGAGCTCAAGCTTGGATGGTGGGAAGATGGATACAGTTGGTGC-3'
```

[SEQ ID NO:13]

with residues 17- 46 hybridizing to the mouse Ck region. For VH PCR, the 3' primers have the degenerate sequences:

```
                                    A       G   T
5'-TATAGAGCTCAAGCTTCCAGTGGATAGACCGATGGGGCTGTCGTTTTGGC-3'
                                    T
```

[SEQ ID NO:14]

with residues 17 - 50 hybridizing to mouse gamma chain CH1. The VL and VH cDNAs were subcloned into pCR4Blunt-TOPO vector (Invitrogen, Carlsbad, CA) for sequence determination. DNA sequencing was carried out by PCR cycle sequencing reactions with fluorescent dideoxy chain terminators (Applied Biosystems, Foster City, CA) according to the manufacturer's instruction. The sequencing reactions were analyzed on a Model 377 DNA Sequencer (Applied Biosystems).

[0111] Construction of humanized 266 (Hu266) variable regions. Humanization of the mouse antibody V regions was carried out as outlined by Queen et al. [*Proc. Natl. Acad. Sci. USA* 86:10029-10033 (1988)]. The human V region framework used as an acceptor for Mu266 CDRs was chosen based on sequence homology. The computer programs ABMOD and ENCAD [Levitt, M., J. Mol. Biol. 168:595-620 (1983)] were used to construct a molecular model of the variable regions. Amino acids in the humanized V regions that were predicted to have contact with CDRs were substituted with the corresponding residues of Mu266. This was done at residues 46, 47, 49, and 98 in the heavy chain and at residue 51 in the light chain. The amino acids in the humanized V region that were found to be rare in the same V-region subgroup were changed to the consensus amino acids to eliminate potential immunogenicity. This was done at residues 42 and 44 in the light chain.

[0112] The light and heavy chain variable region genes were constructed and amplified using eight overlapping synthetic oligonucleotides ranging in length from approximately 65 to 80 bases [He, X. Y., et al., J. Immunol. 160: 029-1035 (1998)]. The oligonucleotides were annealed pairwise and extended with the Klenow fragment of DNA polymerase I, yielding four double-stranded fragments. The resulting fragments were denatured, annealed pairwise, and extended with Klenow, yielding two fragments. These fragments were denatured, annealed pairwise, and extended once again, yielding a full-length gene. The resulting product was amplified by PCR using the Expand High Fidelity PCR System (Roche Molecular Biochemicals, Indianapolis, IN). The PCR-amplified fragments were gel-purified and cloned into pCR4Blunt-TOPO vector. After sequence confirmation, the VL and VH genes were digested with MluI and XbaI, gel-purified, and subcloned respectively into vectors for expression of light and heavy chains to make pVk-Hu266 and pVg1-Hu266 (see Figures 6 and 7, respectively, herein) [Co, M. S., et al., J. Immunol. 148:1149-1154 (1992)]. The mature humanized 266 antibody expressed from these plasmids has the light chain of SEQ ID NO:11 and the heavy chain of SEQ ID NO:12.

[0113] Stable transfection. Stable transfection into mouse myeloma cell line Sp2/0 was accomplished by electroporation using a Gene Pulser apparatus (BioRad, Hercules, CA) at 360 V and 25 μF as described (Co et al., 1992). Before transfection, pVk-Hu266 and pVg1-Hu266 plasmid DNAs were linearized using FspI. Approximately 10^7 Sp2/0 cells were transfected with 20 μg of pVk-Hu266 and 40 μg of pVg1-Hu266. The transfected cells were suspended in DME medium containing 10% FBS and plated into several 96-well plates. After 48 hr, selection media (DME medium containing 10% FBS, HT media supplement, 0.3 mg/ml xanthine and 1 μg/ml mycophenolic acid) was applied. Approximately 10 days after the initiation of the selection, culture supematants were assayed for antibody production by ELISA as shown below. High yielding clones were expanded in DME medium containing 10% FBS and further analyzed for antibody expression. Selected clones were then adapted to growth in Hybridoma SFM.

[0114] Measurement of antibody expression by ELISA. Wells of a 96-well ELISA plate (Nunc-Immuno plate, Cat #

439454, NalgeNunc, Naperville, IL) were coated with 100 µl of 1 µg/ml goat anti-human IgG, Fcγ fragment specific, polyclonal antibodies (Cat # 109-005-098, Jackson ImmunoResearch, West Grove, PA) in 0.2 M sodium carbonate-bicarbonate buffer (pH 9.4) overnight at 4°C. After washing with Washing Buffer (PBS containing 0.1% Tween 20), wells were blocked with 400 µl of Superblock Blocking Buffer (Cat # 37535, Pierce) for 30 min and then washed with Washing Buffer. Samples containing Hu266 were appropriately diluted in ELISA Buffer (PBS containing 1% BSA and 0.1% Tween 20) and applied to ELISA plates (100 µl per well). As a standard, humanized anti-CD33 IgG1 monoclonal antibody HuM195 (Co, *et al.*, 1992, *above*) was used. The ELISA plate was incubated for 2 hr at room temperature and the wells were washed with Wash Buffer. Then, 100 µl of 1/1,000-diluted HRP-conjugated goat anti-human kappa polyclonal antibodies (Cat # 1050-05, Southern Biotechnology, Birmingham, AL) in ELISA Buffer was applied to each well. After incubating for 1 hr at room temperature and washing with Wash Buffer, 100 µl of ABTS substrate (Cat #s 507602 and 506502, Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added to each well. Color development was stopped by adding 100 µl of 2% oxalic acid per well. Absorbance was read at 415 nm using an OPTImax microplate reader (Molecular Devices, Menlo Park, CA).

[0115] Purification of Hu266. One of the high Hu266-expressing Sp2/0 stable transfectants (clone 1D9) was adapted to growth in Hybridoma SFM and expanded to 2 liter in roller bottles. Spent culture supernatant was harvested when cell viability reached 10% or below and loaded onto a protein-A Sepharose column. The column was washed with PBS before the antibody was eluted with 0.1 M glycine-HCl (pH 2.5), 0.1 M NaCl. The eluted protein was dialyzed against 3 changes of 2 liter PBS and filtered through a 0.2 µm filter prior to storage at 4°C. Antibody concentration was determined by measuring absorbance at 280 nm (1 mg/ml =1.4 A_{280}). SDS-PAGE in Tris-glycine buffer was performed according to standard procedures on a 4-20% gradient gel (Cat # EC6025, Novex, San Diego, CA). Purified humanized 266 antibody is reduced and run on an SDS-PAGE gel. The whole antibody shows two bands of approximate molecular weights 25 kDa and 50 kDa. These results are consistent with the molecular weights of the light chain and heavy chain or heavy chain fragment calculated from their amino acid compositions.

Example 14

In vitro binding properties of humanized 266 antibody

[0116] The binding efficacy of humanized 266 antibody, synthesized and purified as described above, was compared with the mouse 266 antibody using biotinylated mouse 266 antibody in a comparative ELISA. Wells of a 96-well ELISA plate (Nunc-Immuno plate, Cat # 439454, NalgeNunc) were coated with 100 µl of β-amyloid peptide (1-42) conjugated to BSA in 0.2 M sodium carbonate/bicarbonate buffer (pH 9.4) (10µg/mL) overnight at 4°C. The $A\beta_{1-42}$-BSA conjugate was prepared by dissolving 7.5 mg of $A\beta_{1-42}$-Cys_{43} (C-terminal cysteine $A\beta_{1-42}$, AnaSpec) in 500 µL of dimethylsulfoxide, and then immediately adding 1,500 µL of distilled water. Two (2) milligrams of maleimide-activated bovine serum albumin (Pierce) was dissolved in 200 µL of distilled water. The two solutions were combined, thoroughly mixed, and allowed to stand at room temperature for two (2) hours. A gel chromatography column was used to separate unreacted peptide from $A\beta_{1-42}$-Cys-BSA conjugate.

[0117] After washing the wells with phosphate buffered saline (PBS) containing 0.1% Tween 20 (Washing Buffer) using an ELISA plate washer, the wells were blocked by adding 300 µL of SuperBlock reagent (Pierce) per well. After 30 minutes of blocking, the wells were washed Washing Buffer and excess liquid was removed.

[0118] A mixture ofbiotinylated Mu266 (0.3 µg/ml final concentration) and competitor antibody (Mu266 or Hu266; starting at 750 µg/ml final concentration and serial 3-fold dilutions) in ELISA Buffer were added in triplicate in a final volume of 100 µl per well. As a no-competitor control, 100 µl of 0.3 µg/ml biotinylated Mu266 was added. As a background control, 100 µl of ELISA Buffer was added. The ELISA plate was incubated at room temperature for 90 min. After washing the wells with Washing Buffer, 100 µl of 1 µg/ml HRP-conjugated streptavidin (Cat # 21124, Pierce) was added to each well. The plate was incubated at room temperature for 30 min and washed with Washing Buffer. For color development, 100 µl/well of ABTS Peroxidase Substrate (Kirkegaard & Perry Laboratories) was added. Color development was stopped by adding 100 µl/well of 2% oxalic acid. Absorbance was read at 415 nm. The absorbances were plotted against the log of the competitor concentration, curves were fit to the data points (using Prism) and the IC50 was determined for each antibody using methods well-known in the art.

[0119] The mean IC50 for mouse 266 was 4.7 µg/mL (three separate experiments, standard deviation =1.3 µg/mL) and for humanized 266 was 7.5 µg/mL (three separate experiments, standard deviation =1.1 µg/mL). A second set of three experiments were carried out, essentially as described above, and the mean IC50 for mouse 266 was determined to be 3.87 µg/mL (SD = 0.12µg/mL) and for human 266, the IC50 was determined to be 4.0 µg/mL (SD = 0.5 µg/mL). On the basis of these results, we conclude that humanized 266 has binding properties that are very similar to those of the mouse antibody 266. Therefore, we expect that humanized 266 has very similar *in vitro* and *in vivo* activities compared with mouse 266 and will exhibit in humans the same effects demonstrated with mouse 266 in mice.

Example 15

In vitro binding properties of mouse antibodies 266 and 4G8

[0120] Antibody affinity (KD = Kd / Ka) was determined using a BIAcore biosensor 2000 and data analyzed with BIAevaluation (v. 3.1) software. A capture antibody (rabbit anti-mouse) was coupled via free amine groups to carboxyl groups on flow cell 2 of a biosensor chip (CM5) using N-ethyl-N-dimethylaminopropyl carbodiimide and N-hydroxysuccinimide (EDC/NHS). A non-specific rabbit IgG was coupled to flow cell 1 as a background control. Monoclonal antibodies were captured to yield 300 resonance units (RU). Amyloid-beta 1-40 or 1-42 (Biosource International, Inc.) was then flowed over the chip at decreasing concentrations (1000 to 0.1 times KD). To regenerate the chip, bound anti-Aβ antibody was eluted from the chip using a wash with glycine-HCl (pH 2). A control injection containing no amyloid-beta served as a control for baseline subtraction. Sensorgrams demonstrating association and dissociation phases were analyzed to determine Kd and Ka. Using this method, the affinity of mouse antibody 266 for both $A\beta_{1-40}$ and for $A\beta_{1-42}$ was found to be 4 pM. The affinity of 4G8 for $A\beta_{1-40}$ was 23 nM and for $A\beta_{1-42}$ was 24 nM. Despite a 6000-fold difference in affinities for Aβ, both 266 and 4G8, which bind to epitopes between amino acids 13 and 28 of Aβ, effectively sequester Aβ from human CSF. Therefore, the location of the epitope is paramount, rather than binding affinity, in determining the ability of an antibody to sequester Aβ and to provide the beneficial and surprising advantages of the present invention.

Example 16

Epitope mapping of mouse antibody 266 using Biacore methodolgy and soluble peptides

[0121] The BIAcore is an automated biosensor system for measuring molecular interactions [Karlsson R., et al. J. Immunol. Methods 145:229-240 (1991)]. The advantage of the BIAcore over other binding assays is that binding of the antigen can be measured without having to label or immobilize the antigen (i.e. the antigen maintains a more native conformation). The BIAcore methodology was used to assess the binding of various amyloid-beta peptide fragments to mouse antibody 266, essentially as described above in Example 12, except that all dilutions were made with HEPES buffered saline containing Tween 20, a variety of fragments of A□(BioSource International) were injected, and a single concentration of each fragment was inj ected (440 nM).

[0122] Amyloid beta fragments 1-28, 12-28, 17-28 and 16-25 bound to mouse antibody 266, while Aβ fragments1-20, 10-20, and 22-35 did not bind. Fragments 1-20, 10-20 and 22-35 bound to other MAbs with known epitope specificity for those regions of Aβ. Using this methodology, the binding epitope for the mouse antibody 266 appears to be between amino acids 17 and 25 of Aβ. Since binding usually occurs with at least 3 residues of the epitope present, one could further infer that the epitope is contained within residues 19-23.

Example 17

In vitro binding properties of humanized antibody 266

[0123] The affinity (KD = Kd / Ka) of humanized 266 antibody, synthesized and purified as described above, was determined essentially as described above in Example 15. Using this method, the affinity of humanized 266 for $A\beta_{1-42}$ was found to be 4 pM.

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Asp Gly Asn Ala Tyr Leu His Trp Phe Leu Gln Lys Pro Gly Gln Ser
        35                  40                  45

Pro Xaa Leu Leu Ile Tyr Lys Val Ser Asn Arg Phe Ser Gly Val Pro
    50                  55                  60

Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile
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Gln Pro Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu Ile Tyr Ser
20 25 30

Asp Gly Asn Ala Tyr Leu His Trp Phe Leu Gln Lys Pro Gly Gln Ser
35 40 45

Pro Arg Leu Leu Ile Tyr Lys Val Ser Asn Arg Phe Ser Gly Val Pro
50 55 60

Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile
65 70 75 80

Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Ser Gln Ser
85 90 95

Thr His Val Pro Trp Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys
100 105 110

Arg Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu
115 120 125

Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe
130 135 140

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Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln
145                 150                 155                 160

Ser Gly Asn Ser Gln Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser
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Thr Tyr Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu
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Lys His Lys Val Tyr Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser
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Pro Val Thr Lys Ser Phe Asn Arg Gly Glu Cys
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Ser Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Leu Val
        35                  40                  45

Ala Gln Ile Asn Ser Val Gly Asn Ser Thr Tyr Tyr Pro Asp Thr Val
    50                  55                  60

Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Leu Tyr
65                  70                  75                  80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Ser Gly Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
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Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Ser Ser Lys
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Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser
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Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser
                165                 170                 175

Leu Ser Ser Val Val Thr Val Pro Ser Ser Ser Leu Gly Thr Gln Thr
            180                 185                 190

Tyr Ile Cys Asn Val Asn His Lys Pro Ser Asn Thr Lys Val Asp Lys
        195                 200                 205

Lys Val Glu Pro Lys Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys
    210                 215                 220

Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro
225                 230                 235                 240

Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys
                245                 250                 255

Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp
            260                 265                 270

Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu
        275                 280                 285

Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu
    290                 295                 300

His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn
305                 310                 315                 320

Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly
                325                 330                 335

Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu
            340                 345                 350

Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr
        355                 360                 365

Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn
    370                 375                 380

Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe
385                 390                 395                 400
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Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn
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1 5 10 15

<210> 16
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Gln Ile Asn Ser Val Gly Asn Ser Thr Tyr Tyr Pro Asp Ser Val Lys
1 5 10 15

Gly

Claims

1. A humanized antibody or fragment thereof that specifically binds an epitope contained within positions 13-28 of Aβ.

2. A humanized antibody or fragment thereof that sequesters Aβ from its bound, circulating form in blood, and alters clearance of soluble and bound forms of Aβ in central the nervous system and plasma.

3. The humanized antibody or fragment thereof of claims 1 or 2 that specifically binds an epitope contained within positions 13-20 of Aβ.

4. The humanized antibody or fragment thereof of claims 1 or 2 that specifically binds an epitope having an amino acid between positions 17-25 of Aβ.

5. The humanized antibody or fragment thereof of claim 4 that specifically binds an epitope having an amino acid between positions 19-23 of Aβ.

6. The humanized antibody or fragment thereof of claim 1 or 2 that specifically binds to the epitope of Aβ peptide to which antibody Mab 266 specifically binds.

7. The humanized antibody or fragment thereof of claim 6, which is humanized Mab 266 or a fragment thereof.

8. The humanized antibody or fragment thereof of claims 4 or 5, wherein the epitope position is determined by the BIAcore method.

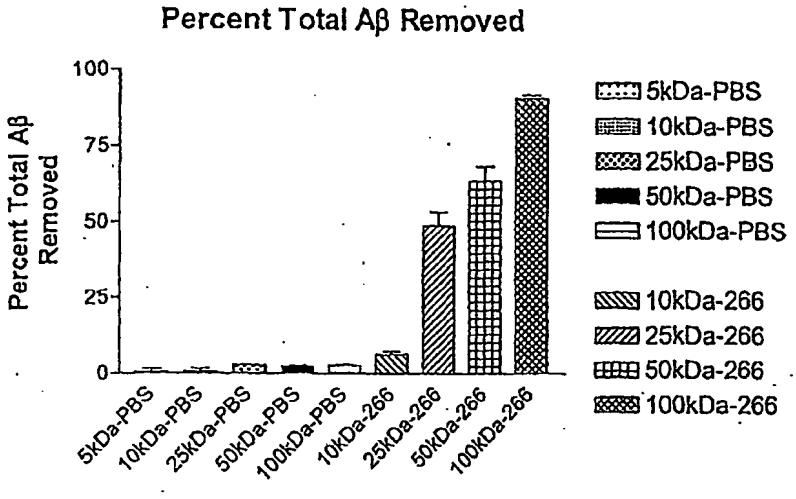
9. A pharmaceutical composition that comprises the humanized antibody or fragment thereof of any one of claims 1-8, and a pharmaceutically acceptable excipient.

10. The use of the humanized antibody or fragment thereof of any one of claims 1-8 for the manufacture of a medicament for inhibiting the formation of amyloid plaques or the effects of toxic soluble Aβ species; reducing amyloid plaques or the effects of toxic soluble Aβ species; preventing, treating or reversing cognitive decline; or improving cognition in a human subject.

11. The use of the humanized antibody or fragment thereof of any one of claims 1-8 for the manufacture of a medicament for preventing or treating clinical or pre-clinical Alzheimer's disease, Down's syndrome, or clinical or pre-clinical cerebral amyloid angiopathy in a human subject.

12. The use of any one of claims 10-11, wherein said antibody or fragment, when administered peripherally to a human subject, need not cross the blood-brain barrier to exert its beneficial effects.

13. The use of any one of claims 10-11, wherein said antibody or fragment, when administered peripherally to a human subject, does not require cellular responses in the subject's brain to exert its beneficial effects.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Percent Total Aβ Removed
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75
50
25
0
5kDa-PBS
10kDa-PBS
25kDa-PBS
50kDa-PBS
100kDa-PBS
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25kDa-266
50kDa-266
100kDa-266
5kDa-PBS
10kDa-PBS
25kDa-PBS
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Figure 1

Aβ Levels in Plasma following Intravenous 266 Injection in APP^{V717F} Mice

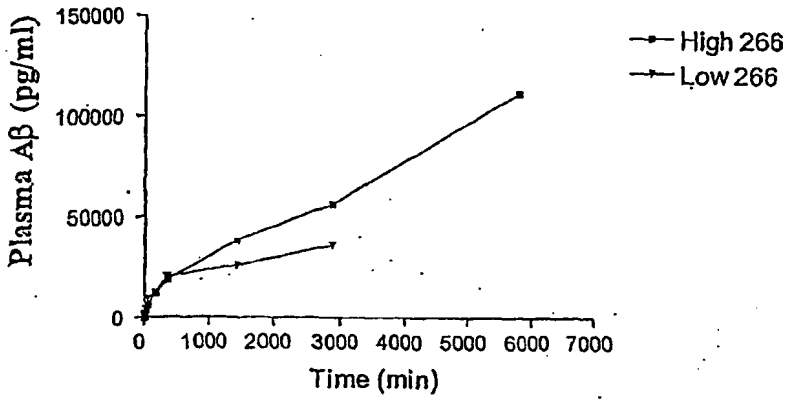

Figure 2

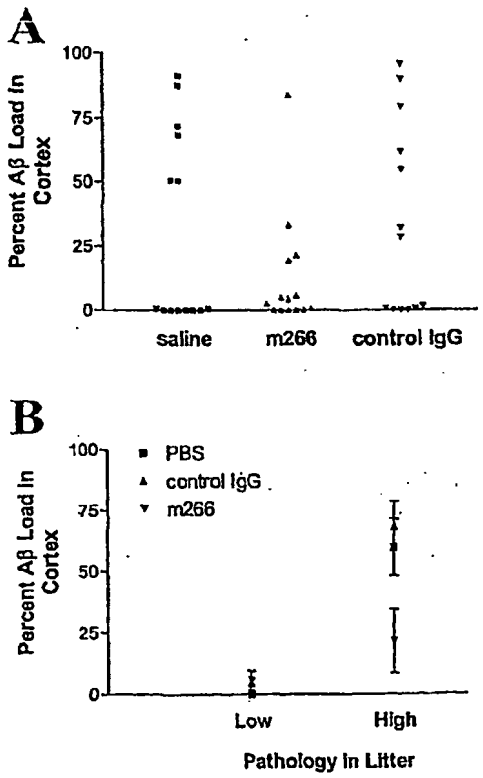
A
Percent Aβ Load in Cortex
100
75
50
25
0
saline
m266
control IgG
B
Percent Aβ Load in Cortex
PBS
control IgG
m266
100
75
50
25
0
Low
High
Pathology in Litter

Figure 3

Figure 4. pVk-Hu266 polynucleotide sequences for expressing humanized 266 light chain and single amino acid codes for expressed humanized 266 light chain

```
619 ACGCGTCCACCATGAAGTTGCCTGTTAGGCTGTTGGTGCTGATGTTCTGGATTCCTGCTTCCAGGTGTGATGTTGTGATG
              M  K  L  P  V  R  L  L  V  L  M  F  W  I  P  A  S  R  C  D  V  V  M

699 ACCCAGAGCCCACTCTCCCTGCCTGTCACCCTTGGACAACCAGCCTCCATCTCTTGCAGATCTAGTCAGAGCCTTATATA
    T  Q  S  P  L  S  L  P  V  T  L  G  Q  P  A  S  I  S  C  R  S  S  Q  S  L  I  Y

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      S  D  G  N  A  Y  L  H  W  F  L  Q  K  P  G  Q  S  P  R  L  L  I  Y  K  V  S  N

859 ACCGATTTTCTGGGGTCCCAGACAGGTTCAGTGGCAGTGGATCTGGGACAGATTTCACACTCAAGATCAGCAGAGTGGAG
  R  F  S  G  V  P  D  R  F  S  G  S  G  S  G  T  D  F  T  L  K  I  S  R  V  E

939 GCCGAGGATGTGGGAGTTTATTACTGTTCTCAAAGTACACATGTTCCGTGGACGTTCGGTCAAGGCACCAAGGTGGAAAT
    A  E  D  V  G  V  Y  Y  C  S  Q  S  T  H  V  P  W  T  F  G  Q  G  T  K  V  E  I

1019 CAAACGTGAGTAGAATTTAATCTAGAAATTCTAAACTCTGAGGGGGTCGGATGACGTGGCCATTCTTTGCCTAAAGCATT
       K  R

1099 GAGTTTACTGCAAGGTCAGAAAAGCATGCAAAGCCCTCAGAATGCCTGCAAAGAGCTCCAACAAAACAATTTAGAACTTT

1179 ATTAAGGAATAGGGGGAAGCTAGGAAGAAACTCAAAACATCAAGATTTTAAATACGCTTCTTGGTCTCCTTGCTATAATT

1259 ATCTGGGATAAGCATGCTGTTTTCTGTCTGTCCCTAACATGCCCTGTGATTATCCGCAAACAACACACCCAAGGGCAGAA

1339 CTTTGTTACTTAAACACCATCCTGTTTGCTTCTTTCCTCAGGAACTGTGGCTGCACCATCTGTCTTCATCTTCCCGCCAT
                                    T  V  A  A  P  S  V  F  I  F  P  P  S

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1579 CCTCAGCAGCACCCTGACGCTGAGCAAAGCAGACTACGAGAAACACAAAGTCTACGCCTGCGAAGTCACCCATCAGGGCC
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1659 TGAGCTCGCCCGTCACAAAGAGCTTCAACAGGGGAGAGTGTTAGAGGGAGAAGTGCCCCCACCTGCTCCTCAGTTCCAGC
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1739 CTGACCCCCTCCCATCCTTTGGCCTCTGACCCTTTTTCCACAGGGGACCTACCCCTATTGCGGTCCTCCAGCTCATCTTT
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2139 CCTCCTCAGCAAGCCCTCATAGTCCTTTTTAAGGGTGACAGGTCTTACAGTCATATATCCTTTGATTCAATTCCCTGAGA
2219 ATCAACCAAAGCAAATTTTTCAAAAGAAGAAACCTGCTATAAAGAGAATCATTCATTGCAACATGATATAAAATAACAAC
2299 ACAATAAAAGCAATTAAATAAACAAACAATAGGGAAATGTTTAAGTTCATCATGGTACTTAGACTTAATGGAATGTCATG
2379 CCTTATTTACATTTTTAAACAGGTACTGAGGGACTCCTGTCTGCCAAGGGCCGTATTGAGTACTTTCCACAACCTAATTT
2459 AATCCACACTATACTGTGAGATTAAAAACATTCATTAAAATGTTGCAAAGGTTCTATAAAGCTGAGAGACAAATATATTC
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2539 TATAACTCAGCAATCCCACTTCTAGGATCC

The complete sequence of the Hu266 light chain gene is located between the MluI and BamHI sites in pVk-Hu266. The nucleotide number indicates its position in pVgk-Hu266. The V_k and C_k exons are translated in single letter code; the dot indicates the translation termination codon. The mature heavy chain starts at the double-underlined aspartic acid (D). The intron sequences are in italic.

Figure 5. pVg1-Hu266 polynucleotide sequences for expressing humanized 266 heavy chain and single amino acid codes for expressed humanized 266 heavy chain

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619 ACGCGTCCACCATGAATTTCGGGCTCAGCTTGATTTTCCTTGTCCTTGTTTTAAAAGGTGTCCTGTGTGAAGTGCAGCTG
          M  N  F  G  L  S  L  I  F  L  V  L  V  L  K  G  V  L  C  E  V  Q  L

699 GTGGAGTCTGGGGGAGGTTTAGTGCAGCCTGGAGGGTCCCTGAGACTCTCCTGTGCAGCCTCTGGATTCACTTTTAGTAG
    V  E  S  G  G  G  L  V  Q  P  G  G  S  L  R  L  S  C  A  A  S  G  F  T  F  S  R

779 GTATTCCATGTCTTGGGTTCGCCAGGCTCCAGGCAAGGGCCTGGAATTGGTCGCACAAATTAATAGTGTTGGTAATAGCA
    Y  S  M  S  W  V  R  Q  A  P  G  K  G  L  E  L  V  A  Q  I  N  S  V  G  N  S

859 CCTACTATCCAGACACTGTAAAGGGCCGATTCACCATCTCCAGAGACAATGCCAAGAACACCCTGTACCTGCAAATGAAC
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     S

1099 AGGTGAGGCAGGTGGCGCCAGCCAGGTGCACACCCAATGCCCATGAGCCCAGACACTGGACGCTGAACCTCGCGGACAGT
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                                                                                    A

1259 CCACCAAGGGCCCATCGGTCTTCCCCCTGGCACCCTCCTCCAAGAGCACCTCTGGGGGCACAGCGGCCCTGGGCTGCCTG
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1339 GTCAAGGACTACTTCCCCGAACCGGTGACGGTGTCGTGGAACTCAGGCGCCCTGACCAGCGGCGTGCACACCTTCCCGGC
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1419 TGTCCTACAGTCCTCAGGACTCTACTCCCTCAGCAGCGTGGTGACCGTGCCCTCCAGCAGCTTGGGCACCCAGACCTACA
       V  L  Q  S  S  G  L  Y  S  L  S  S  V  V  T  V  P  S  S  S  L  G  T  Q  T  Y

1499 TCTGCAACGTGAATCACAAGCCCAGCAACACCAAGGTGGACAAGAAAGTTGGTGAGAGGCCAGCACAGGGAGGGAGGGTG
     I  C  N  V  N  H  K  P  S  N  T  K  V  D  K  K  V

1579 TCTGCTGGAAAGCCAGGCTCAGCGCTCCTGCCTGGACGCATCCCGGCTATGCAGCCCCAGTCCAGGGCAGCAAGGCAGGCC
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1899 TCTCCTCCCAGATTCCAGTAACTCCCAATCTTCTCTCTGCAGAGCCCAAATCTTGTGACAAAACTCACACATGCCCACCG
                                                    E  P  K  S  C  D  K  T  H  T  C  P  P

1979 TGCCCAGGTAAGCCAGCCCAGGCCTCGCCCTCCAGCTCAAGGCGGGACAGGTGCCCTAGAGTAGCCTGCATCCAGGGACA
      C  P

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                                                      A  P  E  L  L  G  G  P  S  V  F  L

2139 TTCCCCCCAAAACCCAAGGACACCCTCATGATCTCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGAGCCACGA
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2219 AGACCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAAAGCCGCGGGAGGAGCAGT
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2299 ACAACAGCACGTACCGTGTGGTCAGCGTCCTCACCGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAAG
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2379 GTCTCCAACAAAGCCCTCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCAAAGGTGGGACCCGTGGGGTGCGAGGGCC
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2459 ACATGGACAGAGGCCGGCTCGGCCCACCCTCTGCCCTGAGAGTGACCGCTGTACCAACCTCTGTCCCTACAGGGCAGCCC
                                                                                G  Q  P

2539 CGAGAACCACAGGTGTACACCCTGCCCCCATCCCGGGATGAGCTGACCAAGAACCAGGTCAGCCTGACCTGCCTGGTCAA
     R  E  P  Q  V  Y  T  L  P  P  S  R  D  E  L  T  K  N  Q  V  S  L  T  C  L  V  K

2619 AGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAACAACTACAAGACCACGCCTCCCG
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      G  P  Y  P  S  D  I  A  V  E  W  E  S  N  G  Q  P  E  N  N  Y  K  T  T  P  P

2699  TGCTGGACTCCGACGGCTCCTTCTTCCTCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTC
      V  L  D  S  D  G  S  F  F  L  Y  S  K  L  T  V  D  K  S  R  W  Q  Q  G  N  V  F

2779  TCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGTCTCCGGGTAAATGAGTGCG
       S  C  S  V  M  H  E  A  L  H  N  H  Y  T  Q  K  S  L  S  L  S  P  G  K  •

2859  ACGGCCGGCAAGCCCCCGCTCCCCGGGCTCTCGCGGTCGCACGAGGATGCTTGGCACGTACCCCCGTACATACTTCCCG
2939  GGCGCCCAGCATGGAAATAAAGCACCCAGCGCTGCCCTGGGCCCCTGCGAGACTGTGATGGTTCTTTCCACGGGTCAGGC
3019  CGAGTCTGAGGCCTGAGTGGCATGAGGGAGGCAGAGCGGGTCCCACTGTCCCCACACTGGCCCAGGCTGTGCAGGTGTGC
3099  CTGGGCCGCCTAGGGTGGGGCTCAGCCAGGGGCTGCCCTCGGCAGGGTGGGGGATTTGCCAGCGTGGCCCTCCCTCCAGC
3179  AGCACCTGCCCTGGGCTGGGCCACGGGAAGCCCTAGGAGCCCCTGGGGACAGACACACAGCCCCTGCCTCTGTAGGAGAC
3259  TGTCCTGTTCTGTGAGCGCCCTGTCCTCCGACCTCCATGCCCACTCGGGGGCATGCCTAGTCCATGTGCGTAGGGACAGG
3339  CCCTCCCTCACCCATCTACCCCCACGGCACTAACCCCTGGCTGCCCTGCCCAGCCTCGCACCCGCATGGGGACACAACCG
3419  ACTCCGGGGACATGCACTCTCGGGCCCTGTGGAGGGACTGGTGCAGATGCCCACACACACACTCAGCCCAGACCCGTTCA
3499  ACAAACCCCGCACTGAGGTTGGCCGGCCACACGGCCACCACACACACACGTGCACGCCTCACACACGGAGCCTCACCCGG
3579  GCGAACTGCACAGCACCCAGACCAGAGCAAGGTCCTCGCACACGTGAACACTCCTCGGACACAGGCCCCCACGAGCCCCA
3659  CGCGGCACCTCAAGGCCCACGAGCCCTCTCGGCAGCTTCTCCACATGCTGACCTGCTCAGACAAACCCAGCCCTCCTCTCA
3739  CAAGGGTGCCCCTGCAGCCGCCACACACACACAGGGGATCACACACCACGTCACGTCCCTGGCCCTGGCCCACTTCCCAG
3819  TGCCGCCCTTCCCTGCAGGATCC
```

The complete sequence of the Hu266 heavy chain gene is located between the MluI and BamHI sites in pVg1-Hu266. The nucleotide number indicates its position in pVg1-Hu266. The V_H and C_H exons are translated in single letter code. The dot indicates the translation termination codon. The mature heavy chain starts at the double-underlined glutamic acid (E). The intron sequences are in italics.

Figure 6. Plasmid pVkHu266

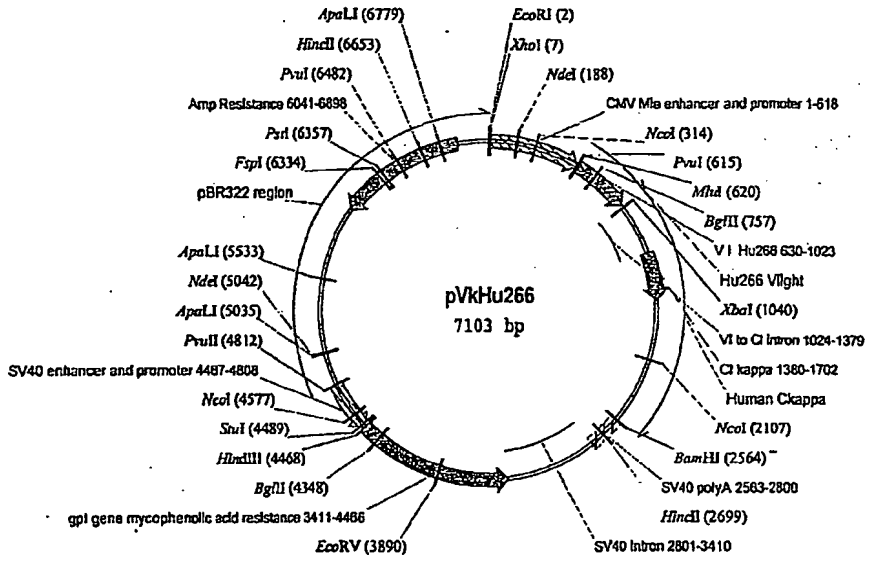
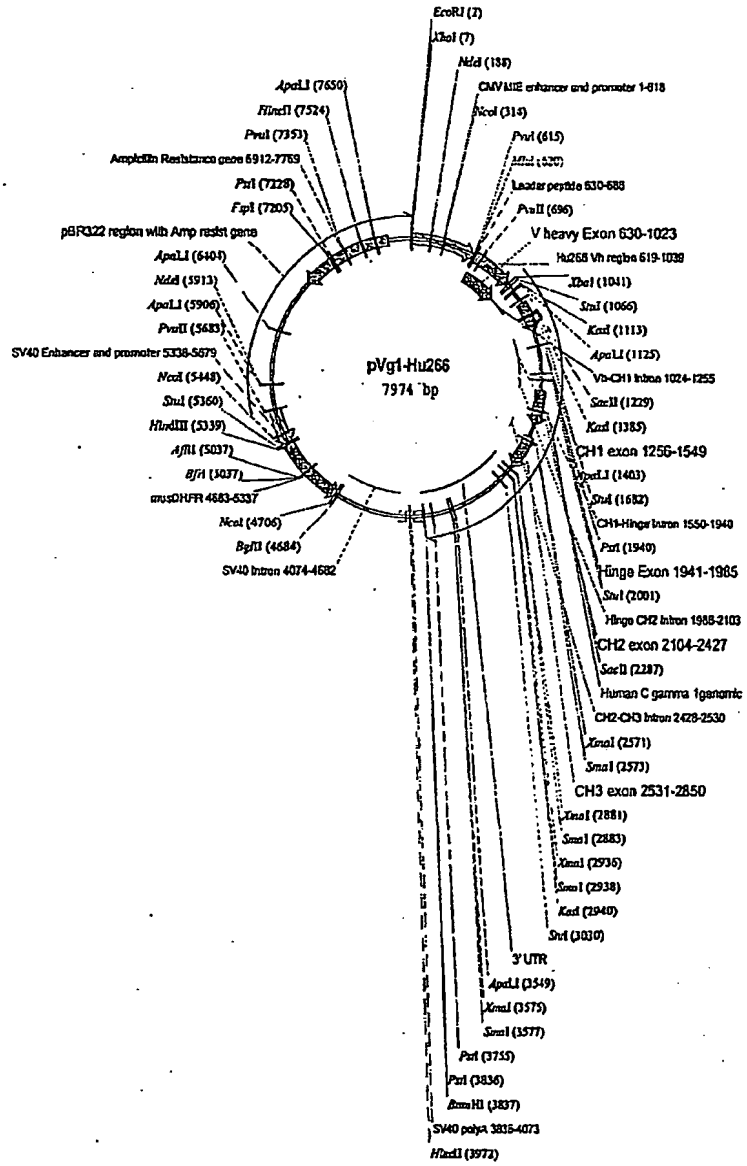


Figure 7 Plasmid pVg1-Hu266



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(54) **Humanized antibodies that sequester amyloid beta peptide**

(57) A method to treat conditions characterized by formation of amyloid plaques both prophylactically and therapeutically is described. The method employs humanized antibodies which sequester soluble Aβ peptide from human biological fluids or which preferably specifically bind an epitope contained within position 13-28 of the amyloid beta peptide Aβ.

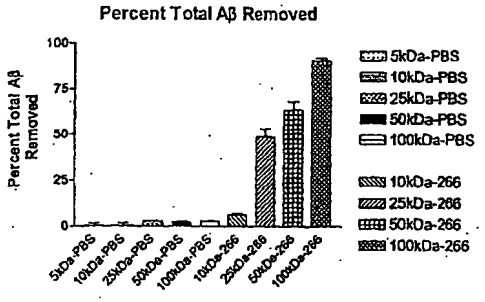

Figure 1

EP 1 481 992 A3

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EUROPEAN SEARCH REPORT

Application Number
EP 04 01 1466

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
Y	WINTER ET AL: "HUMANIZED ANTIBODIES" IMMUNOLOGY TODAY, ELSEVIER PUBLICATIONS, CAMBRIDGE, GB, vol. 14, no. 6, 1996, pages 243-246, XP001005438 ISSN: 0167-4919 * See pages 243-245 (Building humanized antibodies) *	1-13	C07K16/18 C12N15/13 C12N5/10 A61K39/00 A61P25/28
Y	SEUBERT ET AL: "ISOLATION AND QUANTIFICATION OF SOLUBLE ALZHEIMER'S BETA-PEPTIDE FROM BIOLOGICAL FLUIDS" NATURE, MACMILLAN JOURNALS LTD. LONDON, GB, vol. 359, no. 6393, 24 September 1992 (1992-09-24), pages 325-327, XP000616173 ISSN: 0028-0836 * See the antibody 266 *	1-13	
Y	PILLOT ET AL: "Fusogenic properties of the C-terminal domain of the Alzheimer beta-amyloid peptide" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 271, 1996, pages 28757-28765, XP002299386 * See references to the central domain (13-28) throughout the article *	1-13	TECHNICAL FIELDS SEARCHED (Int.Cl.7) C07K C12N A61K A61P
Y	ST GEORGE-HYSLOP ET AL: "Antibody clears senile plaque" NATURE, vol. 400, July 1999 (1999-07), pages 116-117, XP002299786 * See page 116 (clearance of amyloid-beta peptide) *	1-13	

-/--

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
Berlin	8 October 2004	Korsner, S-E

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

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European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 04 01 1466

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
P,Y, D	BARD ET AL: "Peripherally administered antibodies against amyloid beta-peptide enter the central nervous system and reduce pathology in a mouse model of Alzheimer disease" NATURE MEDICINE, NATURE PUBLISHING, CO, US, vol. 6, no. 8, August 2000 (2000-08), pages 916-919, XP002154518 ISSN: 1078-8956 * See the Summary * -----	1-13	

TECHNICAL FIELDS SEARCHED (Int.Cl.7)

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
Berlin	8 October 2004	Korsner, S-E

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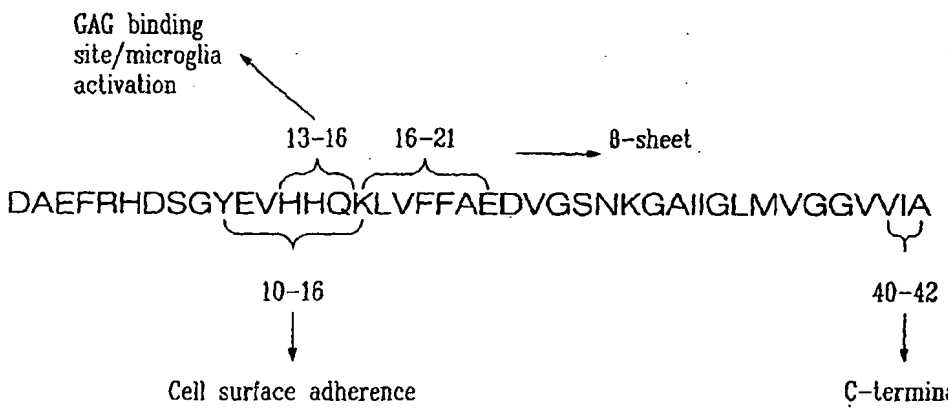
(54) Title: VACCINE FOR THE PREVENTION AND TREATMENT OF ALZHEIMER'S AND AMYLOID RELATED DISEASES



(57) Abstract: The present invention relates to a stereochemically based "non-self" antigen vaccine for the prevention and/or treatment of Alzheimer's and other amyloid related diseases. The present invention provides a vaccine for the prevention and treatment of Alzheimer's and other amyloid related diseases, which overcomes the drawbacks associated with using naturally occurring peptides, proteins or immunogens.

WO 02/096937 A2

VACCINE FOR THE PREVENTION AND TREATMENT OF ALZHEIMER'S AND AMYLOID RELATED DISEASES

BACKGROUND OF THE INVENTION

The present invention relates to a new stereochemically based "non-self" antigen vaccine for the prevention and/or treatment of Alzheimer's and other amyloid related diseases.

Amyloidosis refers to a pathological condition characterized by the presence of amyloid fibers. Amyloid is a generic term referring to a group of diverse but specific protein deposits (intracellular and/or extracellular) which are seen in a number of different diseases. Though diverse in their occurrence, all amyloid deposits have common morphologic properties, stain with specific dyes (*e.g.*, Congo red), and have a characteristic red-green birefringent appearance in polarized light after staining. They also share common ultrastructural features and common x-ray diffraction and infrared spectra.

Amyloid-related diseases can either be restricted to one organ or spread to several organs. The first instance is referred to as "localized amyloidosis" while the second is referred to as "systemic amyloidosis".

Some amyloidotic diseases can be idiopathic, but most of these diseases appear as a complication of a previously existing disorder. For example, primary amyloidosis can appear without any other pathology or can follow plasma cell dyscrasia or multiple myeloma. Secondary amyloidosis is usually seen associated with chronic infection (such as tuberculosis) or chronic inflammation (such as rheumatoid arthritis). A familial form of secondary amyloidosis is also seen in Familial Mediterranean Fever (FMF). This familial type of amyloidosis, as one of the other types of familial amyloidosis, is genetically inherited and is found in

specific population groups. In these two types of amyloidosis, deposits are found in several organs and are thus considered systemic amyloid diseases. Another type of systemic amyloidosis is found in long-term hemodialysis patients. In each of these cases, a different amyloidogenic protein is involved in amyloid deposition.

"Localized amyloidoses" are those that tend to involve a single organ system. Different amyloids are also characterized by the type of protein present in the deposit. For example, neurodegenerative diseases such as scrapie, bovine spongiform encephalitis, Creutzfeldt-Jakob disease and the like are characterized by the appearance and accumulation of a protease-resistant form of a prion protein (referred to as AScr or PrP-27) in the central nervous system. Similarly, Alzheimer's disease, another neurodegenerative disorder, is characterized by neuritic plaques and neurofibrillary tangles. In this case, the plaque and blood vessel amyloid is formed by the deposition of fibrillar Aβ amyloid protein. Other diseases such as adult-onset diabetes (Type II diabetes) are characterized by the localized accumulation of amyloid in the pancreas.

Once these amyloids have formed, there is no known, widely accepted therapy or treatment which significantly dissolves the deposits *in situ*.

Each amyloidogenic protein has the ability to organize into β-sheets and to form insoluble fibrils which get deposited extracellularly or intracellularly. Each amyloidogenic protein, although different in amino acid sequence, has the same property of forming fibrils and binding to other elements such as proteoglycan, amyloid P and complement component. Moreover, each amyloidogenic protein has amino acid sequences which, although different, will show similarities such as regions with the ability to bind to the glycosaminoglycan (GAG) portion of proteoglycan (referred to as the GAG binding site) as well as other regions which will promote β-sheet formation.

In specific cases, amyloidotic fibrils, once deposited, can become toxic to the surrounding cells. As per example, the Aβ fibrils organized as senile plaques have been shown to be associated with dead neuronal cells and microgliosis in patients with Alzheimer's disease. When tested *in vitro*, Aβ peptide was shown to be capable of triggering an activation process of microglia (brain macrophages), which would explain the presence of microgliosis and brain inflammation found in the brain of patients with Alzheimer's disease.

In another type of amyloidosis seen in patients with Type II diabetes, the amyloidogenic protein IAPP has been shown to induce β-islet cell toxicity *in vitro*. Hence, appearance of IAPP fibrils in the pancreas of Type II diabetic patients could contribute to the loss of the β islet cells (Langerhans) and organ dysfunction.

People suffering from Alzheimer's disease develop a progressive dementia in adulthood, accompanied by three main structural changes in the brain: diffuse loss of neurons in multiple parts of the brain; accumulation of intracellular protein deposits termed neurofibrillary tangles; and accumulation of extracellular protein deposits termed amyloid or senile plaques, surrounded by misshapen nerve terminals (dystrophic neurites). A main constituent of these amyloid plaques is the amyloid-β peptide (Aβ), a 40-42 amino-acid protein that is produced through cleavage of the β-amyloid precursor protein (APP). Although symptomatic treatments exist for Alzheimer's disease, this disease cannot be prevented nor cured at this time.

The use of a vaccine to treat Alzheimer's disease is possible in principle (Schenk, D. *et al.*, (1999) *Nature* 400, 173-177). Schenk *et al.* show that, in a transgenic mouse model of brain amyloidosis (as seen in Alzheimer's disease), immunization with Aβ peptide inhibits the formation of amyloid plaques and the associated dystrophic neurites. In that study, a vaccine using the human aggregated all-L peptide as immunogen prevented the formation of β-amyloid plaque, astrogliosis and neuritic dystrophy in vaccinated transgenic mice.

However, it is apparent that there are a number of drawbacks to using an endogenous protein as a vaccine (or a protein naturally present in the animal being vaccinated). Some of these drawbacks include:

- Possible development of autoimmune disease due to the generation of antibodies against "self" protein.
- Difficulty in eliciting an immune response due to the failure of the host immune system to break tolerance.
- Possible development of an acute inflammatory response in the brain due to antibody-mediated phagocytosis by microglia cells.
- Development of anti-idiotype antibodies.

SUMMARY OF THE INVENTION

The present invention relates to a stereochemically based "non-self" antigen vaccine for the prevention and/or treatment of Alzheimer's and other amyloid related diseases. One aim of the present invention is to provide a vaccine for the prevention and treatment of Alzheimer's and other amyloid related diseases, which overcomes the drawbacks associated with using naturally occurring peptides, proteins or immunogens, such as a fibril protein (e.g., beta Amyloid).

The term "fibril and/or amyloid peptide" as used herein, encompasses both monomeric, oligomeric and soluble β-amyloid or any fibril protein, as discussed herein, as well as any other structural variants that may occur naturally, are synthetically constructed or correspond to a known fibril protein. Specifically, a fibril and/or amyloid peptide consists of at least 3 amino acids from a fibril peptide, such as amyloid or any structural variant thereof. The term "corresponds to" is used herein to mean that a polynucleotide sequence is homologous (i.e., is identical, not strictly evolutionarily related) to all or a portion of a reference polynucleotide sequence, or that a polypeptide sequence is identical to a reference polypeptide

sequence. In contradistinction, the term "complementary to" is used herein to mean that the complementary sequence is homologous to all or a portion of a reference polynucleotide sequence. For illustration, the nucleotide sequence "TATAC" corresponds to a reference sequence "TATAC" and is complementary to a reference sequence "GTATA".

The term "amyloid related diseases" includes diseases associated with the accumulation of amyloid either in soluble or insoluble (plaque) forms, which can either be restricted to one organ, "localized amyloidosis", or spread to several organs, "systemic amyloidosis". Secondary amyloidosis may be associated with chronic infection (such as tuberculosis) or chronic inflammation (such as rheumatoid arthritis), including a familial form of secondary amyloidosis which is also seen in Familial Mediterranean Fever (FMF). Another type of systemic amyloidosis found in long-term hemodialysis patients. Localized forms of amyloidosis include, without limitation, diabetes type II and any related disorders thereof, neurodegenerative diseases such as scrapie, bovine spongiform encephalitis, Creutzfeldt-Jakob disease, Alzheimer's disease, Cerebral Amyloid Angiopathy, and prion protein related disorders.

The vaccines of the present invention may be used in the prevention and/or treatment of amyloid related diseases, and in the manufacture of medicaments for preventing and/or treating amyloid-related diseases. A fibril peptide or protein can be derived from a fibril precursor protein known to be associated with certain forms of amyloid diseases, as described herein. Such precursor proteins include, but are not limited to, Serum Amyloid A protein (ApoSAA), immunoglobulin light chain, immunoglobulin heavy chain, ApoAI, transthyretin, lysozyme, fibrinogen α chain, gelsolin, cystatin C, Amyloid beta protein precursor (β-APP), $Beta_2$ microglobulin, prion precursor protein (PrP), atrial natriuretic factor, keratin, islet amyloid polypeptide, a peptide hormone, and synuclein. Such precursors also include mutant proteins, protein fragments and proteolytic peptides of such precursors. In a preferred embodiment, the peptide is effective to induce an immune response

directed against an epitope formed by an amyloidogenic protein or peptide, with respect to a fibril precursor protein. That is, as described in more detail herein, many fibril-forming peptides or proteins are fragments of such precursor proteins, such as those listed above. When such fragments are formed, such as by proteolytic cleavage, epitopes may be revealed that are not present on the precursor and are therefore not immunologically available to the immune system when the fragment is a part of the precursor protein.

In another embodiment, the peptide is effective to induce an immune response directed against an epitope formed by an amyloidogenic protein or peptide. An amyloidogenic protein or peptide is synonymous with an amyloid peptide or protein, and encompasses a protein or peptide which is capable of forming fibrils, plaques, or amyloid deposits. The terms "Aβ," "Aβ peptide", "β-amyloid peptide" and "Amyloidβ" peptide are synonymous, and refer to one or more peptide compositions of about 38-43 amino acids derived from Beta Amyloid Precursor Protein (β-APP), as described herein. Disaggregated Aβ means soluble, monomeric and oligomeric peptide units of Aβ. One method to prepare monomeric Aβ is to dissolve lyophilized peptide in neat DMSO with sonication. The resulting solution is centrifuged to remove any insoluble particulates. Aggregated Aβ is a mixture of oligomers in which the monomeric units are held together by noncovalent bonds. Furthermore, APP^{695}, APP^{751}, and APP^{770} refer, respectively, to the 695, 751, and 770 amino acid residue long polypeptides encoded by the human APP gene. See Kang et al., *Nature* 325, 773 (1987); Ponte et al., *Nature* 331, 525 (1988); and Kitaguchi et al., *Nature* 331, 530 (1988). Amino acids within the human amyloid precursor protein (APP) are assigned numbers according to the sequence of the APP77O isoform. Terms such as Aβ39, Aβ40, Aβ41, Aβ42 and Aβ43 refer, respectively, to an Aβ peptide containing amino acid residues 1-39, 1-40, 1-41, 1-42 and 1-43. Preferably, Aβ peptide contains amino acid residues 10-21. More preferably, the Aβ peptide contains amino acid residues 13-21.

Accordingly, in one embodiment of the present invention, a vaccine is provided which is produced using a "non-self" peptide or protein synthesized from the unnatural D-configuration amino acids, to avoid the drawbacks of using "self" proteins. The peptides need not be aggregated to be operative or immunogenic, in contrast to the prior art vaccines. Thus, an "immunogenic peptide" or "immunogen" or "antigen" is a molecule that is capable of inducing an immunological response against itself upon administration to a patient, either in conjunction with, or in the absence of, an adjuvant. Such molecules include, for example, amyloidogenic peptides or fragments thereof conjugated to a carrier protein, such as keyhole limpet hemocyanin (KLH) , C3d, polysaccharide, bovine serum albumin (BSA), Tetanus toxoid, heat shock protein (HSP), Ovalbumin or cholera toxin.

The term "immunological" or "immune" or "immunogenic" response refers to the development of a humoral (antibody mediated) and/or a cellular (mediated by antigen-specific T cells or their secretion products) response directed against an antigen in a vertebrate individual. Such a response can be an active response induced by administration of immunogen or a passive response induced by administration of antibodies or immune cells such as primed T-cells, B cells, macrophages NK or NKT cells, or primed dendritic cells which can act as antigen presenting cells. A cellular immune response is elicited by the presentation of polypeptide epitopes in association with Class I or Class II MHC molecules to activate antigen-specific $CD4^+$ T helper cells and/or $CD8^+$ cytotoxic T cells. The response may also involve activation of immune cells, such as monocytes, macrophages, NK cells, basophils, dendritic cells, astrocytes, microglia cells, eosinophils or other components of innate immunity. The presence of a cell-mediated immunological response can be determined by standard proliferation assays ($CD4^+$ T cells) or CTL (cytotoxic T lymphocyte) assays known in the art. The relative contributions of humoral and cellular responses to the protective or therapeutic effect of an immunogen can be distinguished by separately isolating immunoglobulin (IgG) and T-cell fractions

from an immunized first mammal and measuring a protective or therapeutic effect in a second subject.

The terms "polynucleotide" and "nucleic acid," as used interchangeably herein refer to a polymeric molecule having a backbone that supports bases capable of hydrogen bonding to typical polynucleotides, where the polymer backbone presents the bases in a manner to permit such hydrogen bonding in a sequence specific fashion between the polymeric molecule and a typical polynucleotide (e.g., single-stranded DNA). Such bases are typically inosine, adenosine, guanosine, cytosine, uracil and thymidine. Polymeric molecules include double and single stranded RNA and DNA, and backbone modifications thereof, for example, methylphosphonate linkages. The term "polypeptide" as used herein refers to a compound made up of a single chain of amino acid residues linked by peptide bonds. The term "protein" may be synonymous with the term "polypeptide" or may refer to a complex of two or more polypeptides. The term "peptide" also refers to a compound composed of amino acid residues linked by peptide bonds. Generally peptides are composed of 100 or fewer amino acids, while polypeptides or proteins have more than 100 amino acids. As used herein, the term "protein fragment" may also be read to mean a peptide.

Except as otherwise expressly defined herein, the abbreviations used herein for designating the amino acids and the protective groups are based on recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (*Biochemistry*, 1972, 11:1726-1732).

In another embodiment, there is provided a method for preventing and/or treating an amyloid-related disease in a subject, which features administering to the subject an antigenic amount of an all-D peptide which elicits production of antibodies against the all-D peptide, and elicits an immune response by the subject, therefore preventing fibrillogenesis, associated cellular toxicity and neurodegeneration, wherein the antibodies interact with at least one region of an

amyloid protein, a fibril protein or another non-amyloid protein which induces amyloidosis. A "fibril peptide" or "fibril protein" refers to a monomeric or oligomeric or aggregated form of a protein or peptide that forms fibrils present in amyloid plaques. Examples of such peptides and proteins are provided herein. "Nonamyloid protein" containing formulations include, but are not limited to: compositions that produce immune responses against gelsolin fragments for treatment of hereditary systemic amyloidosis, mutant lysozyme protein (Alys), for treatment of a hereditary neuropathy, mutant alpha chain of fibrinogen (AfibA) for a non-neuropathic form of amyloidosis manifest as renal disease, and mutant cystatin C (Acys) for treatment of a form of hereditary cerebral angiopathy reported in Iceland. In addition, certain hereditary forms of prion disease (e.g., Creutzfeldt-Jacob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome (GSS), and fatal familial insomnia (FFI)) are characterized by a mutant isoform of prion protein, PrP^{Sc}. This protein can be used in therapeutic compositions for treatment and prevention of deposition of PrP plaques, in accordance with the present invention. These vaccines may be used in the prevention and/or treatment of amyloid related diseases, and in the manufacture of medicaments for preventing and/or treating amyloid-related diseases.

In a further embodiment of the invention, a vaccine for preventing and/or treating an amyloid-related disease in a subject comprises at least one antibody or fragment thereof which interacts with amyloid proteins to prevent fibrillogenesis, wherein the antibodies are raised against an antigenic amount of an all-D peptide of such a protein, *e.g.*, β sheet region, GAG-binding site region, Aβ (1-42, all-D) and macrophage adherence region Aβ (10-16), immunogenic fragments thereof, protein conjugates thereof, immunogenic derivative peptides thereof, immunogenic peptides thereof, and immunogenic peptidomimetics thereof, or a peptide which has a substantial identity to any of the above peptides.

The following terms are used to describe the sequence relationships between two or more polynucleotides: "substantial identity", "comparison window",

"sequence identity", "percentage of sequence identity", and "reference sequence." A "reference sequence" is a defined sequence used as a basis for a sequence comparison; a reference sequence may be a subset of a larger sequence, for example, as a segment of a full-length cDNA or gene sequence given in a sequence listing, or may comprise a complete cDNA or gene sequence. Generally, a reference sequence is at least 20 nucleotides in length, frequently at least 25 nucleotides in length, and often at least 50 nucleotides in length. Since two polynucleotides may each (1) comprise a sequence (i.e., a portion of the complete polynucleotide sequence) that is similar between the two polynucleotides, and (2) may further comprise a sequence that is divergent between the two polynucleotides, sequence comparisons between two (or more) polynucleotides are typically performed by comparing sequences of the two polynucleotides over a "comparison window" to identify and compare local regions of sequence similarity. A "comparison window", as used herein, refers to a conceptual segment of at least 20 contiguous nucleotide positions wherein a polynucleotide sequence may be compared to a reference sequence of at least 20 contiguous nucleotides and wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) of 20 percent or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Optimal alignment of sequences for aligning a comparison window may be conducted by the local homology algorithm of Smith and Waterman (1981) Adv. Appl. Math. 2: 482, by the homology alignment algorithm of Needleman and Wunsch (1970) J. Mol. Biol. 48: 443, by the search for similarity method of Pearson and Lipman (1988) Proc. Natl. Acad. Sci. (U.S.A.) 85: 2444, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Dr., Madison, Wis.), or by inspection, and the best alignment (i.e., resulting in the highest percentage of homology over the comparison window) generated by the various methods is selected.

The term "sequence identity" means that two polynucleotide sequences are identical (i.e., on a nucleotide-by-nucleotide basis) over the window of comparison. The term "percentage of sequence identity" is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (e.g., A, T, C, G, U, or I) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (i.e., the window size), and multiplying the result by 100 to yield the percentage of sequence identity. The terms "substantial identity" as used herein denotes a characteristic of a polynucleotide sequence, wherein the polynucleotide comprises a sequence that has at least 85 percent sequence identity, preferably at least 90 to 95 percent sequence identity, more usually at least 99 percent sequence identity as compared to a reference sequence over a comparison window of at least 20 nucleotide positions, frequently over a window of at least 25-50 nucleotides, wherein the percentage of sequence identity is calculated by comparing the reference sequence to the polynucleotide sequence which may include deletions or additions which total 20 percent or less of the reference sequence over the window of comparison.

As applied to polypeptides, the term "substantial identity" means that two peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights, share at least 80 percent sequence identity, preferably at least 90 percent sequence identity, more preferably at least 95 percent sequence identity or more (e.g., 99 percent sequence identity). Preferably, residue positions which are not identical differ by conservative amino acid substitutions. Conservative amino acid substitutions refer to the interchangeability of residues having similar side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of

amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids having sulfur-containing side chains is cysteine and methionine. Preferred conservative amino acid substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, and asparagine-glutamine.

The term "antibody" or "immunoglobulin" is used to include intact antibodies and fragments thereof. An antibody can be a monoclonal or polyclonal antibody and can be made by recombinant techniques, collected from serum or ascites, or from hybridoma sources. Typically, fragments compete with the intact antibody from which they were derived for specific binding to an antigen fragment. Fragments are produced by recombinant DNA techniques, or by enzymatic or chemical cleavage of intact antibodies. Antigen binding fragments include Fab, Fab', $F(ab')_2$, Fv, and single-chain antibodies, further including separate heavy chains or light chains. Antibody fragments are produced by recombinant DNA techniques, or by enzymatic or chemical separation of intact immunoglobulins. The term "antibody" encompasses one or more immunoglobulin chains that are chemically conjugated to, or expressed as, fusion proteins with other proteins. The term "antibody" also includes bispecific antibodies. A bispecific or bifunctional antibody is an artificial hybrid antibody having two different heavy/light chain pairs and two different binding sites. Bispecific antibodies can be produced by a variety of methods including fusion of hybridomas or linking of Fab' fragments. See, e.g., Songsivilai & Lachmann, *Clin. Exp. Immunol.* 79:315-321 (1990); Kostelny et al., *J. Immunol.* 148, 1547-1553 (1992).

As used herein, "Single-chain Fv" or "sFv" antibody fragments comprise the VH and VL domains of antibody, wherein these domains are present in a single polypeptide chain. Preferably, the Fv polypeptide further comprises a polypeptide linker between the VH and VL domains which enables the sFv to form the desired structure for antigen binding. For a review of sFv *see*, Pluckthun, The

Pharmacology of Monoclonal Antibodies. vol. 113. Rosenburg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994).

Specific binding between two entities means an affinity of at least 10^6 M^{-1}, 10^7 M^{-1}, 10^8 M^{-1}, 10^9M^{-1}, or $10^{10}M^{-1}$. Affinities greater than 10^8 M^{-1} are preferred. As discussed herein, minor variations in the amino acid sequences of antibodies or immunoglobulin molecules are contemplated as being encompassed by the present invention, providing that the variations in the amino acid sequence maintain at least 75%, more preferably at least 80%, 90%, 95%, and most preferably 99%. In particular, conservative amino acid replacements are contemplated. Conservative replacements are those that take place within a family of amino acids that are related in their side chains. Genetically encoded amino acids are generally divided into families: (1) acidic=aspartate, glutamate; (2) basic=lysine, arginine, histidine; (3) non-polar=alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan; and (4) uncharged polar=glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine. More preferred families are: serine and threonine are aliphatic-hydroxy family; asparagine and glutamine are an amide-containing family; alanine, valine, leucine and isoleucine are an aliphatic family; and phenylalanine, tryptophan, and tyrosine are an aromatic family. For example, it is reasonable to expect that an isolated replacement of a leucine with an isoleucine or valine, an aspartate with a glutamate, a threonine with a serine, or a similar replacement of an amino acid with a structurally related amino acid will not have a major effect on the binding or properties of the resulting molecule, especially if the replacement does not involve an amino acid within a framework site. Whether an amino acid change results in a functional peptide can readily be determined by assaying the specific activity of the polypeptide derivative. Assays are described in detail herein. Preferred amino- and carboxy-termini of fragments or analogs occur near boundaries of functional domains. Structural and functional domains can be identified by comparison of the nucleotide and/or amino acid sequence data to public or proprietary sequence databases. Preferably, computerized comparison methods are used to identify sequence motifs or predicted protein conformation domains that occur in other

proteins of known structure and/or function. Methods to identify protein sequences that fold into a known three-dimensional structure are known. Bowie et al. *Science* 253:164 (1991). Thus, the foregoing examples demonstrate that those of skill in the art can recognize sequence motifs and structural conformations that may be used to define structural and functional domains in accordance with the invention. Preferred amino acid substitutions are those which: (1) reduce susceptibility to proteolysis, (2) reduce susceptibility to oxidation, (3) alter binding affinity for forming protein complexes, (4) alter binding affinities, and (4) confer or modify other physicochemical or functional properties of such analogs. Analogs can include various muteins of a sequence other than the naturally-occurring peptide sequence. For example, single or multiple amino acid substitutions (preferably conservative amino acid substitutions) may be made in the naturally-occurring sequence (preferably in the portion of the polypeptide outside the domain(s) forming intermolecular contacts). A conservative amino acid substitution should not substantially change the structural characteristics of the parent sequence (e.g., a replacement amino acid should not tend to break a helix that occurs in the parent sequence, or disrupt other types of secondary structure that characterizes the parent sequence). Examples of art-recognized polypeptide secondary and tertiary structures are described in *Proteins, Structures and Molecular Principles* (Creighton, Ed., W. H. Freeman and Company, New York (1984)); *Introduction to Protein Structure* (C. Branden and J. Tooze, eds., Garland Publishing, New York, N.Y. (1991)); and Thornton et at. *Nature* 354:105 (1991), which are each incorporated herein by reference.

As used herein, "Humanized" forms of non-human (e.g., rodent) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', $F(ab')_2$, or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues

from a hypervariable region of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity, and capacity. In some instances, framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues which are found neither in the recipient antibody nor in the donor antibody. These modifications are made to further refine and maximize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details. see. Jones et al.. Nature, 3221:5222 525 (1986); Reichmarm et al., Nature, 332:323-329 (1988); and Presta, Curr. Op. Struct. Biol., 2:593-596 (1992).

The term "conjugate" or "conjugated to" is intended to refer to joined together, coupled, bonded and/or fused. A "protein conjugate" or "conjugated protein" is generally intended to refer to a compound of a protein with a non-protein. In addition to the amino acids, a "conjugated protein" contains permanently associated chemical components. The non-amino acid part of the conjugated protein is generally referred to as its prosthetic group. Conjugated proteins are classified on the basis of the chemical nature of their prosthetic groups (Lehninger Principles of Biochemistry, 3d ed. Worth Publishers 2000), which is incorporated by reference herein. In a conjugate vaccine, an antigenic molecule is covalently linked to a "carrier" protein or polypeptide. The linkage serves to increase the antigenicity of the conjugated molecule. Methods for forming conjugate vaccines from an antigenic molecule and a "carrier" protein or polypeptide are known in the art (Jacob, C. O, et al., Eur. J. Immunol. 16:1057-1062 (1986); Parker J. M. R. et al., In: Modem Approaches to Vaccines, Chanock, R. M. et al., eds, pp. 133-138, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1983); Zurawski, V. R, et al., J.

Immunol. 121:122-129 (1978); Klipstein, F. A, et al., Infect. Immun. 37:550-557 (1982); Bessler, W. G, Immunobiol. 170:239-244 (1985); Posnett, D. N, et al., J. Biol. Chem. 263:1719-1725 (1988); Ghose, A. C, et al., Molec. Immunol. 25:223-230 (1988); all of which references are incorporated herein by reference).

Conjugation of a protein and a polysaccharide can provide other advantageous results. For example, it has been found that protein-polysaccharide conjugates enhance the antibody response not only to the polysaccharide component, but also to the protein component. This effect is described, for example, in the dual conjugate patent application of Mond and Lees, U.S. Pas Nos. 5,955,079 and 5,585,100, both of which are incorporated herein by reference. This effect also is described in A. Lees, et al., "Enhanced Immunogenicity of Protein-Dextran Conjugates: I. Rapid Stimulation of Enhanced Antibody Responses to Poorly Immunogenic Molecules," Vaccine, Vol. 12, No. 13, (1994), pp. 1160-1166. This article is entirely incorporated herein by reference. Examples of carrier proteins include, but are not limited to keyhole limpet hemocyanin (KLH) , C3d, polysaccharide, bovine serum albumin (BSA), Tetanus toxoid, heat shock protein (HSP), Ovalbumin or cholera toxin.

In another embodiment, there is provided a method for preventing and/or treating an amyloid-related disease in a subject which comprises administering a pharmaceutical composition comprising a combination of at least two antibodies, or fragments thereof that bind to two or more portions of a fibril protein and/or a nonamyloid protein. In another embodiment the pharmaceutical composition further comprises immune cells, more preferably primed lymphocytes.

A "pharmaceutical composition" refers to a chemical or biological composition suitable for administration to a mammalian individual, preferably a human. Such compositions may be specifically formulated for administration via one or more of a number of routes, including but not limited to, oral, parenteral, intravenous, intraarterial, subcutaneous, intranasal, sublingual, intraspinal, intracerebroventricular, transdermal and the like. A "pharmaceutical excipient" or a "pharmaceutically acceptable excipient" is a carrier, usually a liquid, in which an

active therapeutic peptide is formulated. The excipient generally does not provide any pharmacological activity to the formulation, though it may provide chemical and/or biological stability, release characteristics, and the like. Exemplary formulations can be found, for example, in Remington's Pharmaceutical Sciences, 19th Ed., Grennaro, A., Ed., 1995.

Still in a further embodiment, there is provided a method for preventing and/or treating an amyloid-related disease in a subject, which comprises administering to the subject an antigenic amount of an all-D peptide which, may further interact with at least one region of an amyloid protein, *e.g.*, β sheet region, GAG-binding site region, Aβ (1-42) and macrophage adherence region Aβ (10-16), immunogenic fragments thereof, protein conjugates thereof, immunogenic derivative peptides thereof, immunogenic peptides thereof, and immunogenic peptidomimetics thereof, wherein the compound elicits an immune response by the subject and therefore prevents fibrillogenesis.

According to another related aspect, the invention includes a method of preventing or treating a disorder characterized by amyloid deposition in a mammalian subject. In accordance with this aspect of the invention, the subject is given a dosage of a peptide effective to produce an immune response against an amyloid peptide characteristic of the amyloid disorder from which the subject suffers. Essentially, the methods include administering pharmaceutical compositions containing immunogenic amyloid peptides specific to the disorder, such as those described above. Such methods are further characterized by their effectiveness in inducing immunogenic responses in the subject. According to a preferred embodiment, the method is effective to produce an immunological response that is characterized by a serum titer of at least 1:1000 with respect to the amyloid peptide against which the immunogenic peptide is directed. In yet a further preferred embodiment, the serum titer is at least 1:5000 with respect to the amyloid component. According to a related embodiment, the immune response is characterized by a serum amount of immunoreactivity corresponding to greater than

about four times higher than a serum level of immunoreactivity measured in a pre-treatment control serum sample. This latter characterization is particularly appropriate when serum immunoreactivity is measured by ELISA techniques, but can apply to any relative or absolute measurement of serum immunoreactivity. According to a preferred embodiment, the immunoreactivity is measured at a serum dilution of about 1:100 to 1:10,000 to determine antibody titer.

According to a still further related aspect, the invention includes a method of determining the prognosis of a patient undergoing treatment for an amyloid disorder. Here, patient serum amount of immunoreactivity against an amyloid peptide characteristic of the selected disorder is measured, and a patient serum amount of immunoreactivity of at least four times a baseline control level of serum immunoreactivity is indicative of a prognosis of improved status with respect to the particular amyloid disorder. According to preferred embodiments, the amount of immunoreactivity against the selected amyloid peptide present in the patient serum is characterized by a serum titer of at least about 1:1000, or at least 1:5000, with respect to the amyloid peptide.

"Passive immunization" as used herein refers to the administration of antibodies, fragments thereof, or immune cells, i.e. T-cells B-cells, NK cells, NKT cells , dendritic cells, macrophages, basophils, monocytes, or components of the complement pathway, to an individual in order to confer immunity. Components of the complement pathway can be conjugated to proteins to enhance the inate immune response in combination with active or passive immunization. Immunoglobulins (Ig) obtained from human blood may contain antibodies to a variety of agents depending on the pool of human plasma used in preparation. Specific immunoglobulins are obtained from plasma from donors with high levels of antibodies to specific antigens, or donors immunized to produce such a response (Immunization, Cecil Textbook of Medicine, 19th ed. Vol. 1, W.B. Saunders Company 1992; Harrison's Principles of Internal Medicine, 14th ed, McGraw Hill, 1998). Humanized monoclonal antibodies to sequester amyloid-β peptide in plasma,

brain and cerebrospinal fluid to prevent accumulation of the amyloid-β peptide within the brain and the cerebrovasculature is described in WO01/62801, which is incorporated herein by reference. EP0613007 describes antibodies having specificity for β-amyloid peptide which is predominantly in a β-sheet conformation. Such antibodies are useful for the invention a described herein.

According to one embodiment of the invention, methods and compositions are provided for passive immunization of a mammal in order to prevent or treat amyloid diseases. According to this aspect of the invention, patients are given an effective dosage of an antibody or fragment thereof, that specifically binds to a selected region of amyloid peptide, preferably a region close to or within the central core region of the peptide which is responsible for β-sheet formation or are given immune cells, preferably primed immune cells or antigen presenting cells. In general, such antibodies are selected for their abilities to specifically bind the various proteins, peptides, and components described with respect to the pharmaceutical compositions and methods described in the preceding paragraphs of this section. According to a related embodiment, such methods and compositions may include combinations of antibodies that bind at least two amyloid components. In general, pharmaceutical compositions are administered to provide a serum amount of immunoreactivity against the target amyloid peptide that is at least about four times higher than a serum level of immunoreactivity against the component measured in a control serum sample. The antibodies may also be administered with a carrier, as described herein. In general, in accordance with this aspect of the invention, such antibodies will be administered (or formulated for administration) peritoneally, orally, intranasally, subcutaneously, intramuscularly, transdermally, topically or intravenously, but can be administered or formulated for administration by any pharmaceutically effective route (i.e., effective to produce the indicated therapeutic levels, as set forth above and herein).

The term "epitope" or "antigenic determinant" refers to a site on an antigen to which an antibody or antibody binding fragment specifically binds or to which B

and/or T cells respond. B-cell epitopes can be formed both from contiguous amino acids or noncontiguous amino acids juxtaposed by tertiary folding of a protein. Epitopes formed from contiguous amino acids are typically retained on exposure to denaturing solvents whereas epitopes formed by tertiary folding are typically lost on treatment with denaturing solvents. An epitope typically includes at least 3, and more usually, at least 5 or 8-10 amino acids in a unique spatial conformation. Methods of determining spatial conformation of epitopes include, for example, x-ray crystallography and 2-dimensional nuclear magnetic resonance. See, e.g., *Epitope Mapping Protocols in Methods in Molecular Biology,* Vol. 66, Glenn E. Morris, Ed. (1996). Antibodies that recognize the same epitope can be identified in a simple immunoassay showing the ability of one antibody to block the binding of another antibody to a target antigen. L-cells recognize continuous epitopes of about nine amino acids for CD8 cells or about 13-15 amino acids for CD4 cells. T cells that recognize the epitope can be identified by *in vitro* assays that measure antigen-dependent proliferation, as determined by ^{3}H-thymidine incorporation by primed T cells in response to an epitope (Burke et al., *J. Inf Dis.* 170, 1110-19 (1994)), by antigen-dependent killing (cytotoxic T lymphocyte assay, Tigges et al., *J. Immunol.* 156, 3901-3910) or by cytokine secretion.

As used herein, the term "compound" refers to a peptide of the present invention or a pharmaceutically acceptable composition containing a peptide according to the present invention. In a preferred embodiment of the present invention, the compound is a compound of Formula I:

R'-(P)-R'' **(I)**,

wherein

P is an all-D peptide of an amyloid protein, *e.g.*, β sheet region, GAG-binding site region, Aβ (1-42, all-D), and macrophage adherence region (10-16, all-D), immunogenic fragments thereof, immunogenic derivatives thereof, protein conjugates thereof,

immunogenic peptides thereof, and immunogenic peptidomimetics thereof;

R′ is an N-terminal substituent, *e.g.*:

- hydrogen;
- lower alkyl groups, *e.g.*, acyclic or cyclic having 1 to 8 carbon atoms, without or with functional groups, *e.g.*, carboxylate, sulfonate and phosphonate;
- aromatic groups;
- heterocyclic groups; and
- acyl groups, *e.g.*, alkylcarbonyl, arylcarbonyl, sulfonyl and phosphonyl groups; and

R″ is a C-terminal substituent, *e.g.*, hydroxy, alkoxy, aryloxy, unsubstituted or substituted amino groups.

In an embodiment, R′ and R″ are identical or different, wherein the alkyl or aryl groups of R′ and R″ are further substituted with functionalities such as halide (*e.g.*, F, Cl, Br, and I), hydroxyl, alkoxyl, aryloxyl, hydroxycarbonyl, alkoxylcarbonyl, aryloxycarbonyl, carbamyl, unsubstituted or substituted amino, sulfo or alkyloxysulfonyl, phosphono or alkoxyphosphonyl groups.

When the compound has an acid functional group, it can be in the form of a pharmaceutically acceptable salt or ester. When the compound has a basic functional group, it can be in the form of a pharmaceutically acceptable salt.

In one embodiment, P is a peptide capable of interacting with at least one region of an amyloid protein.

In a preferred embodiment of the present invention, the subject is a human being.

In yet another embodiment of the present invention, the amyloid related disease may be Alzheimer's disease.

In still another embodiment of the present invention, the amyloid related disease may be Cerebral Amyloid Angiopathy (CAA).

In another embodiment of the present invention, there is provided a method for preventing and/or treating an amyloid related disease in a subject, comprising administering to the subject an antigenic amount of a compound of Formula I:

$$R'\text{-}(P)\text{-}R'' \quad (I),$$

wherein

P is an all-D peptide of an amyloid protein, *e.g.*, β sheet region, GAG-binding site region, Aβ (1-42, all-D), and macrophage adherence region (10-16, all-D), immunogenic fragments thereof, immunogenic derivatives thereof, protein conjugates thereof, immunogenic peptides thereof, and immunogenic peptidomimetics thereof;

R' is an N-terminal substituent selected from the group consisting of:

- hydrogen;
- lower alkyl groups, *e.g.*, acyclic or cyclic having 1 to 8 carbon atoms, without or with functional groups, *e.g.*, carboxylate, sulfonate and phosphonate;
- aromatic groups;
- heterocyclic groups; and
- acyl groups, *e.g.*, alkylcarbonyl, arylcarbonyl, sulfonyl and phosphonyl groups; and

R″ is a C-terminal substituent, *e.g.*, hydroxy, alkoxy, aryloxy, unsubstituted or substituted amino groups.

In accordance with this method, the compound elicits an immune response by the subject preventing fibrillogenesis, neurodegeneration or cellular toxicity.

In accordance with a preferred embodiment of the present invention, there is provided a vaccine for preventing and/or treating an amyloid-related disease in a subject, comprising an antigenic amount of an all-D peptide which may further interact with at least one region of an amyloid protein, *e.g.*, β sheet region (16-21), GAG-binding site region (13-16), and macrophage adherence region (10-16, all-D), immunogenic fragments thereof, protein conjugates thereof, immunogenic derivative peptides thereof, immunogenic peptides thereof, and immunogenic peptidomimetics thereof, wherein the compound elicits an immune response by the subject or prevents fibrillogenesis or favors clearance of amyloid protein prior to fibril formation. In one aspect, the vaccine alters soluble Aβ levels in the plasma of a mammal thereby decreasing soluble Aβ levels in the brain of a mammal, and thereby preventing the formation of fibril plaques.

In another embodiment, some methods entail passive immunization against soluble Aβ or fibrillary Aβ. In one aspect, the invention provides a method for preventing and/or treating an amyloid-related disease in a first mammal in need thereof, comprising administering an antigenic amount of an all-D amyloid-β peptide to a second mammal, wherein said all-D amyloid-β peptide induces an immune response against the amyloid-β peptide in the second mammal, recovering the antibodies or immune cells from the second mammal, and providing a therapeutically or prophylactically effective dose of the antibodies or immune cells to the first mammal. In this embodiment the all-D amyloid-β peptide has an amino acid sequence that is at least 80% identical to a sequence selected from the group consisting of SEQ ID NOS: 1-70. Other reactive epitopes of Aβ useful for the generating antibodies or immune cells according to the present invention include an

epitope within residues 1-42 of Aβ, an epitope within residues 16-21 of Aβ, an epitope within residues 13-21 of Aβ, an epitope within residues 10-21 of Aβ, an epitope within residues 10-16 of Aβ, an epitope within residues 25-35 of Aβ, and an epitope comprising a free N – or C-terminal residue of Aβ. In one aspect, passive immunization against Aβ is effectuated by transference of an immune cell, i.e., a primed lymphocyte such as a B or T cell, from a second mammal actively immunized against Aβ as described, to a first mammal thereby ameliorating or preventing Aβ related disorders in the first mammal. In another aspect, passive immunization against Aβ is effectuated by transference of an antibody or fragment thereof capable of binding specifically to Aβ. In yet another aspect a human, humanized or chimeric antibody is administered to the first mammal thereby ameliorating or preventing Aβ related disorders.

In one embodiment, the present invention is directed to a method for preventing and/or treating an amyloid-related disease in a subject, comprising: administering to the subject an antigenic amount of an all-D amyloid-β peptide, wherein said all-D amyloid-β peptide induces an immune response by said subject against said amyloid-β peptide, and wherein said all-D amyloid-β peptide has an amino acid sequence that is at least 80% identical to a sequence selected from the group consisting of SEQ ID NOS: 1-70 or least one antibody or fragment that specifically binds to an epitope within residues 1-42 of Aβ, an epitope within residues 16-21 of Aβ, an epitope within residues 13-21 of Aβ, an epitope within residues 10-21 of Aβ, an epitope within residues 10-16 of Aβ, an epitope within residues 25-35 of Aβ, and an epitope comprising a free N- or C-terminal residue of Aβ.

In one embodiment, the present invention is directed to altering the serum levels of Aβ in a mammal in need thereof, for example a human, by administering a therapeutic or prophylactic amount of at least one antibody or fragment that specifically binds to an epitope within residues 1-42 of Aβ, an epitope within residues 16-21 of Aβ, an epitope within residues 13-21 of Aβ, an epitope within

residues 10-21 of Aβ, an epitope within residues 10-16 of Aβ, an epitope within residues 25-35 of Aβ, and an epitope comprising a free N- or C-terminal residue of Aβ. In one aspect, altering serum Aβ levels in the mammal alters the levels of soluble Aβ or fibril Aβ in the mammal. In yet another aspect, altering serum Aβ levels in the mammal alters the levels of soluble or fibril Aβ levels in the brain of the mammal. In another aspect altering serum Aβ levels in the mammal favors the clearance of Aβ levels in the brain of a mammal.

The methods can be used on both asymptomatic patients and those currently showing symptoms of disease. The antibody administered in such methods can be a human, humanized, chimeric or nonhuman antibody, or fragment thereof and can be from monoclonal or polyclonal sources, obtained from a mammal, or produced by recombinant techniques. In some methods, the antibody is prepared from a human immunized with Aβ peptide. In other methods, a humanized antibody is administered.

In some methods, the antibody is administered with a pharmaceutical carrier as a pharmaceutical composition. In some methods, antibody is administered at a dosage of 0.0001 to 100 mg/kg, preferably, at least 1 mg/kg body weight antibody. In some methods, the antibody is administered in multiple dosages over a prolonged period, for example, of at least six months. In some methods, the antibody is administered as a sustained release composition. The antibody can be administered, for example, intraperitoneally, orally, subcutaneously, intracranially, intramuscularly, topically, intranasally, transdermally or intravenously.

Other features and advantages of the invention will be apparent from the following detailed description and claims.

BRIEF DESCRIPTION OF THE DRAWING

FIG. 1 illustrates the targeted sites for the antigenic fragments.

FIG. 2 illustrates the effect of 1 mg/ml of polyclonal antibodies raised against D and L forms of Aβ(16-21) on fibrillogenesis.

FIG. 3 illustrates the effect of 0.5 mg/ml of polyclonal antibodies raised against D and L forms of Aβ(16-21) on fibrillogenesis.

FIGs. 4A to 4C illustrate electron micrographs showing the effect of anti-D KLVFFA peptide antibodies (FIG. 4B) and anti-L KLVFFA peptide antibodies (FIG. 4C) with respect to a control (FIG. 4A) on fibrillogenesis.

FIGs. 5A to 5D illustrate the immunohistochemistry of anti-D KLVFFA on aggregated Aβ peptide in brain sections of retrosplenial cortex (FIG. 5A) and parietal cortex (FIG. 5C) and the histochemistry (Thioflavin S assay) of anti-D KLVFFA on aggregated Aβ peptide in the same brain sections of retrospinal cortex (FIG. 5B) and parietal cortex (FIG. 5D).

FIGs. 6A to 6D illustrate the immunohistochemistry of anti-L KLVFFA antibodies on aggregated Aβ peptide in brain sections of parietal cortex (FIG. 6A) and entorrhinal cortex (FIG. 6C) and the histochemistry (Thioflavin S assay) of anti-L KLVFFA antibodies on aggregated Aβ peptide in the same brain sections of parietal cortex (FIG. 6B) and entorrhinal cortex (FIG. 6D).

FIG. 7 illustrates the response of rabbits to KLH-conjugated all-L and all-D KLVFFA.

FIGs. 8A and 8B illustrate the reduction of Aβ40 level, soluble and insoluble fraction in the brain of TgCRND8 mice immunized with D13-21-KLH.

Fig. 9 illustrates the reduction of Aβ42 level, soluble fraction in the brain of TgCRND8 mice immunized with D13-21-KLH.

FIGs. 10A and 10B illustrate the reduction of total Aβ level (Aβ40 and Aβ42) in the brain of TgCRND8 mice immunized with D13-21-KLH.

DETAILED DESCRIPTION OF THE INVENTION

Amyloid diseases or amyloidoses include a number of disease states having a wide variety of outward symptoms. These disorders have in common the presence of abnormal extracellular deposits of protein fibrils, known as "amyloid deposits" or "amyloid plaques" that are usually about 10-100nm in diameter and are localized to specific organs or tissue regions. Such plaques are composed primarily of a naturally occurring soluble protein or peptide which aggregate in insoluble fibrillar deposits. These insoluble deposits are composed of generally lateral aggregates of fibrils that are approximately 10-15 nm in diameter. Amyloid fibrils produce a characteristic apple green birefringence in polarized light, when stained with Congo Red dye.

The peptides or proteins forming the plaque deposits are often produced from a larger precursor protein. More specifically, the pathogenesis of amyloid fibril deposits generally involves proteolytic cleavage of an "abnormal" precursor protein into fragments. These fragments generally aggregate into anti-parallel β-pleated sheets; however, certain undegraded forms of precursor protein have been reported to aggregate and form fibrils in familial amyloid polyneuropathy (variant transthyretin fibrils) and dialysis-related amyloidosis (β_2 microglobulin fibrils) (Tan, S.Y. and Pepys, M.B. Amyloidosis. Histopathology 25: 403-414 (1994)). Specifically, the Aβ (16-21) site is known to play an important role in initiating the harmful process of Aβ peptide amyloidogenesis. It is also known that when these peptides are made from D-amino acids, they retain their ability to interact with the natural all-L-homologous sequence, thereby preventing amyloidogenesis. Other amyloid proteins which may be used in the present invention include, without

limitation, the beta sheet regions of IAPP (e.g. 24-29, all-D), β2-microglobulin, amyloid A protein, and prion-related proteins.

The disorders are traditionally classified on the basis of the major fibril components forming the plaque deposits, as discussed below. However, without being restricted to theory, there is evidence supporting Aβ can be transported back and forth between the brain and the blood (Ghersi-Egea, J., et al., J. Neurochem., 67:880-883, (1996)) and that Aβ in plaques (insoluble or fibrillar Aβ) is in equilibrium with soluble Aβ (Kawarabayashi, T., et al., J. Neurosci. 21:372-381, (2001), both incorporated herein by reference in their entirety. Thus, the identification of a disorder based on the major fibril component is provided for convenience, and amelioration of the disease can be effectuated according to the compositions and methods described herein by targeting either the fibrillar Aβ or the soluble Aβ. The targeting of either the fibrillar Aβ or the soluble Aβ can be achieved by active or passive immunization as described herein and in PCT publications WO 01/62801, WO 01/90182, WO 01/18169, WO 00/77178, WO 00/72880, WO 00/72876, WO 99/60024 and WO 99/27944, each incorporated by reference in its entirety. Levels of targeted Aβ, specifically levels of the soluble Aβ can be determined by methods known in the art and disclosed herein (*see,* U.S. patents 5,766,846, 5,837,672, and 5,593,846, each incorporated by reference in their entirety). These levels can be monitored during the course of prophylactic or therapeutic treatments. A therapeutic endpoint is reached when the disease state is reduced.

Amyloid Diseases

The present invention is based on the discovery that amyloid diseases can be treated by administering peptides that serve to stimulate an immune response against a component or components of the various disease-specific amyloid deposits. The sections below serve to exemplify major forms of amyloidosis and are not intended to limit the invention.

AA (reactive) Amyloidosis

Generally, AA amyloidosis is a manifestation of a number of diseases that provoke a sustained acute phase response. Such diseases include chronic inflammatory disorders, chronic local or systemic microbial infections, and malignant neoplasms.

AA fibrils are generally composed of 8000 dalton fragments (AA peptide or protein) formed by proteolytic cleavage of serum amyloid A protein (ApoSAA), a circulating apolipoprotein which is present in HDL complexes and which is synthesized in hepatocytes in response to such cytokines as IL-1, IL-6 and TNF. Deposition can be widespread in the body, with a preference for parenchymal organs. The spleen is usually a deposition site, and the kidneys may also be affected. Deposition is also common in the heart and gastrointestinal tract.

AA amyloid diseases include, but are not limited to inflammatory diseases, such as rheumatoid arthritis, juvenile chronic arthritis, ankylosing spondylitis, psoriasis, psoriatic arthropathy, Reiter's syndrome, Adult Still's disease, Behcet's syndrome, and Crohn's disease. AA deposits are also produced as a result of chronic microbial infections, such as leprosy, tuberculosis, bronchiectasis, decubitus ulcers, chronic pyelonephritis, osteomyelitis, and Whipple's disease. Certain malignant neoplasms can also result in AA fibril amyloid deposits. These include such conditions as Hodgkin's lymphoma, renal carcinoma, carcinomas of gut, lung and urogenital tract, basal cell carcinoma, and hairy cell leukemia.

AL Amyloidoses

AL amyloid deposition is generally associated with almost any dyscrasia of the B lymphocyte lineage, ranging from malignancy of plasma cells (multiple myeloma) to benign monoclonal gammopathy. At times, the presence of amyloid deposits may be a primary indicator of the underlying dyscrasia.

Fibrils of AL amyloid deposits are composed of monoclonal immunoglobulin light chains or fragments thereof. More specifically, the fragments are derived from the N-terminal region of the light chain (kappa or lambda) and contain all or part of the variable (V_L) domain thereof. Deposits generally occur in the mesenchymal tissues, causing peripheral and autonomic neuropathy, carpal tunnel syndrome, macroglossia, restrictive cardiomyopathy, arthropathy of large joints, immune dyscrasias, myelomas, as well as occult dyscrasias. However, it should be noted that almost any tissue, particularly visceral organs such as the heart, may be involved.

Hereditary Systemic Amyloidoses

There are many forms of hereditary systemic amyloidoses. Although they are relatively rare conditions, adult onset of symptoms and their inheritance patterns (usually autosomal dominant) lead to persistence of such disorders in the general population. Generally, the syndromes are attributable to point mutations in the precursor protein leading to production of variant asnyloidogenic peptides or proteins. Table 1 summarizes the fibril composition of exemplary forms of these disorders.

Table 1

Fibril Peptide/Protein	*Genetic variant*	*Clinical Syndrome*
Transthyretin and fragments (ATTR)	Met30, many others	Familial amyloid polyneuropahty (FAP), (Mainly peripheral nerves)
Transthyretin and fragments (ATTR)	Thr45, Ala60, Ser84, Met111, Ile122	Cardiac involvement predominant without neuropathy
N-terminal fragment of Apolipoprotein A1 (apoAI)	Arg26	Familial amyloid polyneurophathy (FAP), (mainly peripheral nerves)
N-terminal fragment of Apoliproprotein A1 (AapoAI)	Arg26, Arg50, Arg 60, others	Ostertag-type, non-neuropathic (predominantly visceral involvement)

Lysozyme (Alys)	Thr56, His67	Ostertag-type, non-neuropathic (predominantly visceral involvement)
Fibrogen ∀ chain fragment	Leu554, Val 526	Cranial neuropathy with lattic corneal dystrophy
Gelsolin fragment (Agel)	Asn187, Tyr187	Cranial neuropathy with lattice cornealk dystrophy
Cystatin C fragment	Glu68	Hereditary cerebral hemmorrhage (cerebral amyloid angiopathy) – Icelandic type
β-amyloid protein (aβ) derived from Amyloid Precursor Protein (APP)	Gln693	Hereditary cerebral hemmorrhage (cerebral amyloid angiopathy) – Dutch type
β-amyloid protein (aβ) derived from Amyloid Precursor Protein (APP)	Ile717, Phe717, Gly717	Familial Alzheimer's Disease
β-amyloid protein (aβ) derived from Amyloid Precursor Protein (APP)	Asn670, Leu671	Familial Dementia – probably Alzheimer's Disease
Prion Protein (PrP) derived from Prp precursor protein 51-91 insert	Leu102, Val167, Asn178, Lys200	Familial Creutzfeldt-Jakob disease; Gerstmann-Sträussler-Scheinker syndrome (hereditary spongiform encephalopathies, prion diseases)
AA derived from Serum amyloid A protein (ApoSAA)		Familial Mediterranean fever, predominant renal involvement (autosomal recessive)
AA derived from Serum amyloid A protein (ApoSAA)		Muckle-Well's syndrome, nephropathy, deafness, urticaria, limb pain
Unknown		Cardiomyophathy with persistent atrial standstill
Unknown		Cutaneous deposits (bullous, papular, pustulodermal)

*Data derived from Tan & Pepys, 1994, supra.

The data provided in Table 1 are exemplary and are not intended to limit the scope of the invention. For example, more than 40 separate point mutations in the transthyretin gene have been described, all of which give rise to clinically similar forms of familial amyloid polyneuropathy.

Transthyretin (TTR) is a 14 kilodalton protein that is also sometimes referred to as prealbumin. It is produced by the liver and choroid plexus, and it functions in transporting thyroid hormones and vitamin A. At least 50 variant forms of the protein, each characterized by a single amino acid change, are responsible for various forms of familial amyloid polyneuropathy. For example, substitution of proline for leucine at position 55 results in a particularly progressive form of neuropathy; substitution of methionine for leucine at position 111 resulted in a severe cardiopathy in Danish patients. Amyloid deposits isolated from heart tissue of patients with systemic amyloidosis have revealed that the deposits are composed of a heterogeneous mixture of TTR and fragments thereof, collectively referred to as ATTR, the full length sequences of which have been characterized. ATTR fibril components can be extracted from such plaques and their structure and sequence determined according to the methods known in the art (e.g., Gustavsson, A., *et al.*, Laboratory Invest. 73: 703-708, 1995; Kametani, F., *et al.*, Biochem. Biophys. Res. Commun. 125: 622-628, 1984; Pras, M., et al., PNAS 80: 539-42, 1983).

Persons having point mutations in the molecule apolipoprotein Al (e.g., Gly→ Arg26; Trp 4→ Arg50; Leu→ 4 Arg60) exhibit a form of amyloidosis ("Östertag type") characterized by deposits of the protein apolipoprotein Al or fragments thereof (AApoAl). These patients have low levels of high density lipoprotein (HDL) and present with a peripheral neuropathy or renal failure.

A mutation in the alpha chain of the enzyme lysozyme (e.g., Ile→Thr56 or Asp→His57) is the basis of another form of Östertag-type non-neuropathic hereditary amyloid reported in English families. Here, fibrils of the mutant lysozyme protein (Alys) are deposited, and patients generally exhibit impaired renal function.

This protein, unlike most of the fibril-forming proteins described herein, is usually present in whole (unfragmented) form (Benson, M.D., et al. CIBA Fdn. Symp. 199: 104-131, 1996).

β-amyloid peptide (Aβ) is a 39-43 amino acid peptide derived by proteolysis from a large protein known as β Amyloid Precursor protein (βAPP). Mutations in βAPP result in familial forms of Alzheimer's disease, Down's syndrome and/or senile dementia, characterized by cerebral deposition of plaques composed of Aβ fibrils and other components, which are described in further detail below. Known mutations in APP associated with Alzheimer's disease occur proximate to the cleavage sites of β or gamma-secretase, or within Aβ. For example, position 717 is proximate to the site of gamma-secretase cleavage of APP in its processing to Aβ, and positions 670/671 are proximate to the site of β-secretase cleavage. Mutations at any of these residues may result in Alzheimer's disease, presumably by causing an increase the amount of the 42/43 amino acid form of Aβ generated from APP. The structure and sequence of Aβ peptides of various lengths are well known in the art. Such peptides can be made according to methods known in the art (e.g., Glenner and Wong, Biochem Biophys. Res. Comm. 129: 885-890, 1984; Glenner and Wong, Biochem Biophys. Res. Comm. 122: 113 1-1135, 1984). In addition, various forms of the peptides are commercially available.

Synuclein is a synapse-associated protein that resembles an apolipoprotein and is abundant in neuronal cytosol and presynaptic terminals. A peptide fragment derived from alpha-synuclein, termed NAC, is also a component of amyloid plaques of Alzheimer's disease. (Clayton, et al., 1998). This component also serves as a target for immunologically-based treatments of the present invention, as detailed below.

Gelsolin is a calcium binding protein that binds to fragments and actin filaments. Mutations at position 187 (e.g., Asp→ Asn; Asp→ Tyr) of the protein result in a form of hereditary systemic amyloidosis, usually found in patients from

Finland, as well as persons of Dutch or Japanese origin. In afflicted individuals, fibrils formed from gelsolin fragments (Agel), usually consist of amino acids 173-243 (68 kDa carboxyterminal fragment) and are deposited in blood vessels and basement membranes, resulting in corneal dystrophy and cranial neuropathy which progresses to peripheral neuropathy, dystrophic skin changes and deposition in other organs. (Kangas, H., *et al.* Human Mol. Genet. 5(9): 1237-1243, 1996).

Other mutated proteins, such as mutant alpha chain of fibrinogen (AfibA) and mutant cystatin C (Acys) also form fibrils and produce characteristic hereditary disorders. AfibA fibrils form deposits characteristic of a nonneuropathic hereditary amyloid with renal disease; Acys deposits are characteristic of a hereditary cerebral amyloid angiopathy reported in Iceland. (Isselbacher, Ct at., Harrison's Principles of Internal Medicine, McGraw-Hill, San Francisco, 1995; Benson, *et al., supra.*). In at least some cases, patients with cerebral amyloid angiopathy (CAA) have been shown to have amyloid fibrils containing a non-mutant form of cystatin C in conjunction with beta protein. (Nagai, A., *et al.* Molec. Chem. Neuropathol. 33: 63-78, 1998).

Certain forms of prion disease are now considered to be heritable, accounting for up to 15% of cases, which were previously thought to be predominantly infectious in nature. (Baldwin, *et al.*, in Research Advances in *Alzheimer's Disease and Related Disorders*, John Wiley and Sons, New York, 1995). In such prion disorders, patients develop plaques composed of abnormal isoforms of the normal prion protein (PrP^{Sc}). A predominant mutant isoform, PrP^{Sc}, also referred to as AScr, differs from the normal cellular protein in its resistance to protease degradation, insolubility after detergent extraction, deposition in secondary lysosomes, post-translational synthesis, and high β-pleated sheet content. Genetic linkage has been established for at least five mutations resulting in Creutzfeldt-Jacob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome (GSS), and fatal familial insomnia (FFI). (Baldwin) Methods for extracting fibril peptides from scrapie fibrils, determining

sequences and making such peptides are known in the art. (e.g., Beekes, M., *et al.* J. Gen. Virol. 76: 2567-76, 1995).

For example, one form of GSS has been linked to a PrP mutation at codon 102, while telencephalic GSS segregates with a mutation at codon 117. Mutations at codons 198 and 217 result in a form of GSS in which neuritic plaques characteristic of Alzheimer's disease contain PrP instead of Aβ peptide. Certain forms of familial CJD have been associated with mutations at codons 200 and 210; mutations at codons 129 and 178 have been found in both familial CJD and FFI. (Baldwin, *supra*).

Senile Systemic Amyloidosis

Amyloid deposition, either systemic or focal, increases with age. For example, fibrils of wild type transthyretin (TTR) are commonly found in the heart tissue of elderly individuals. These may be asymptomatic, clinically silent, or may result in heart failure. Asymptomatic fibrillar focal deposits may also occur in the brain (Aβ), corpora amylacea of the prostate (β_2 microglobulin), joints and seminal vesicles.

Cerebral Amyloidosis

Local deposition of amyloid is common in the brain, particularly in elderly individuals. The most frequent type of amyloid in the brain is composed primarily of Aβ peptide fibrils, resulting in dementia or sporadic (non-hereditary) Alzheimer's disease. In fact, the incidence of sporadic Alzheimer's disease greatly exceeds forms shown to be hereditary. Fibril peptides forming these plaques are very similar to those described above, with reference to hereditary forms of Alzheimer's disease (AD).

Dialysis-related Amyloidosis

Plaques composed of β_2 microglobulin (β_2M) fibrils commonly develop in patients receiving long term hemodialysis or peritoneal dialysis. β_2 microglobulin is a 11.8 kilodalton polypeptide and is the light chain of Class I MHC antigens, which are present on all nucleated cells. Under normal circumstances, it is continuously shed from cell membranes and is normally filtered by the kidney. Failure of clearance, such as in the case of impaired renal function, leads to deposition in the kidney and other sites (primarily in collagen-rich tissues of the joints). Unlike other fibril proteins, β_2M molecules are generally present in unfragmented form in the fibrils. (Benson, *supra*).

Hormone-derived Amyloidoses

Endocrine organs may harbor amyloid deposits, particularly in aged individuals. Hormone-secreting tumors may also contain hormone-derived amyloid plaques, the fibrils of which are made up of polypeptide hormones such as calcitonin (medullary carcinoma of the thyroid), islet amyloid polypeptide (amylin; occurring in most patients with Type II diabetes), and atrial natriuretic peptide (isolated atrial amyloidosis). Sequences and structures of these proteins are well known in the art.

Miscellaneous Amyloidoses

There are a variety of other forms of amyloid disease that are normally manifest as localized deposits of amyloid. In general, these diseases are probably the result of the localized production and/or lack of catabolism of specific fibril precursors or a predisposition of a particular tissue (such as the joint) for fibril deposition. Examples of such idiopathic deposition include nodular AL amyloid, cutaneous amyloid, endocrine amyloid, and tumor-related amyloid.

Pharmaceutical Compositions

The pharmaceutical compositions of the present invention are directed to vaccines prepared from fibril peptides that have at least 50% of their amino acid

residues in the dextro form (D-isomers) . Preferably, the vaccines are prepared from all D-Aβ (10-21), D-Aβ (13-21), D-Aβ (25-35), D-Aβ (16-21), D-Aβ (10-16), D-Aβ (1-40), D-Aβ (1-42), D-Aβ (10-22), D- Aβ (13-22), or D- Aβ (16-22), or the N – or C-terminal region of D-Aβ(1-42). The vaccines are believed to elicit an immune response in the host or to produce antibodies that recognize the naturally occurring target. As used herein, "all-D" includes peptides having at least 50% D-configuration amino acids. Preferably, "all-D" also includes peptides having greater than or equal to 50%; 55%; 60%; 65%; 70%;75%; 80%; 85%; 90%; 95% or 100% D-configuration amino acids.

The vaccine according to the present invention is able to prevent the development of brain amyloidosis through two possible scenarios: 1) the effect of anti-Aβ antibodies at the site of amyloid deposition, and 2) the systemic effect of the high circulatory anti-Aβ level on the plasmatic Aβ concentrations.

Specifically, elevated plasma anti-Aβ antibody levels may act systemically by decreasing normal Aβ plasma levels, thereby creating a systemic imbalance in the normal Aβ levels. Such an imbalance could lead to activation of the mechanism responsible for clearing Aβ from the brain and into the periphery, in order to re-establish the normal balance between brain and plasma Aβ levels.

Accordingly, this possibility could be exploited by determining the effect of active or passive immunization on plasma and brain Aβ40 levels at different timepoints following such immunization. Aβ-immunization can also exert a systemic protective effect against the development of brain amyloidosis. The ratio of Aβ levels in plasma and brain should remain constant in immunized transgenic animals, while it should decrease in control animals. Additionally, B-cell or bone marrow cell transfer from immunized to naïve transgenic animals should have the same effect as passive immunization using anti-Aβ antibodies.

Furthermore, the vaccine of the present invention does not present the drawbacks of using "self" proteins and does not need to be aggregated to induce an immune response. For example, the antibodies raised against the all-D-Aβ(16-21) peptide can be expected to recognize the all-L-Aβ(16-21) peptide sequence. Pharmaceutical compositions of the present invention may include, in addition to the immunogenic peptide(s), an effective amount of an adjuvant and/or an excipient. Pharmaceutically effective and useful adjuvants and excipients are well known in the art, and are described in more detail below.

According to the present invention, compositions capable of eliciting or providing an immune response directed to certain components of amyloid plaques are effective to treat or prevent development of amyloid diseases. In particular, according to the invention provided herein, it is possible to prevent progression of, ameliorate the symptoms of, and/or reduce the amyloid deposition process in afflicted individuals, when an immunostimulatory dose of an anti-amyloid peptide, or corresponding anti-amyloid immune peptide, is administered to the patient. This section describes exemplary anti-amyloid peptides that produce active, as well as passive, immune responses to amyloid protein and provides exemplary data showing the effect of treatment using such compositions on amyloid brain concentration.

Anti-Amyloid Peptides: Antibodies, Analogs and Fragments of Amyloid Proteins

Generally, anti-amyloid peptides of the invention are composed of a specific plaque component, preferably an amyloid or amyloidogenic component, which is usually a characteristic protein, peptide, or fragment thereof. The human forms of Aβ are referred to as Aβ39, Aβ4O, Aβ41, Aβ42 and Aβ43. The sequences of these peptides and their relationship to the APP precursor are illustrated by Fig. 1 of Hardy et al., TINS 20, 155-158 (1997). For example, Aβ42 has the sequence:

H_2N-Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-
His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-

Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-Val-Gly-Gly-Val-Val-Ile-Ala-OH (SEQ ID NO:1)

Aβ41, Aβ40 and Aβ39 differ from Aβ42 by the omission of Ala, Ala-Ile and Ala-Ile-Val respectively from the C-terminal end. Aβ43 differs from Aβ42 by the presence of a threonine residue at the C-terminus.

Immunogenic fragments of Aβ are advantageous relative to the intact molecule in the present methods for several reasons. First, because only certain epitopes within Aβ induce a useful immunogenic response for treatment of Alzheimer's disease, an equal dosage of mass of a fragment containing such epitopes provides a greater molar concentration of the useful immunogenic epitopes than a dosage of intact Aβ. Second, certain immunogenic fragments of Aβ generate an immunogenic response against amyloid protein without generating a significant immunogenic response against APP protein from which Aβ derives. Third, fragments of Aβ are simpler to manufacture than intact Aβ due to their shorter size. Fourth, fragments of Aβ do not aggregate in the same manner as intact Aβ, simplifying preparation of pharmaceutical compositions and administration thereof. Fifth, full-length Aβ is toxic to cells, but fragments are not, and Aβ aggregates could act as seeds to accelerate plaque deposition, but non-aggregated fragments would not. Sixth, Aβ has the unusual property that it can fix and activate both classical and alternate complement cascades. In particular, it binds to Clq and ultimately to C3bi. This association facilitates binding to macrophages leading to activation of B cells. In addition, C3bi breaks down further and then binds to CR2 on B cells in a T cell dependent manner leading to a 10,000 fold increase in activation of these cells. This mechanism causes Aβ to generate an immune response in excess of that of other antigens (Bradt et al., *Complement-dependent Proinflammatory Properties of the Alzheimer's Disease β-Peptide*, J. Exp. Med. Vol.188, pp. 431-438 (1998); Thornton et al., Clin. Exp. Immunol. Vol. 104, pp. 531-7 (1996); Jacquier-Sarlin et al., Immunology vol. 84, pp. 164-70 (1995).

Some immunogenic fragments of Aβ have a sequence of at least 2, 3, 5, 6, 10 or 20 contiguous amino acids from a natural peptide. Some immunogenic fragments have no more than 10, 9, 8, 7, 5 or 3 contiguous residues from Aβ. Preferred immunogenic fragments include residues 1-42 of Aβ. In some methods, the antibody specifically binds to an epitope within residues 16-21 of Aβ. In some methods, the antibody specifically binds to an epitope within residues 13-21 of Aβ. In some methods, the antibody specifically binds to an epitope within residues 10-21 of Aβ. In some methods, the antibody specifically binds to an epitope within residues 10-16 of Aβ. In some methods, the antibody specifically binds to an epitope within residues 25-35 of Aβ. The designation Aβ16-21 for example, indicates a fragment including residues 16-21 of Aβ and lacking other residues of Aβ. Other less preferred fragments include Aβ 1-5, 1-6, 1-7, 1-10, 3-7, 1-3, and 1-4. These fragments require screening for activity in clearing or preventing amyloid deposits as described in the Examples before use. Fragments lacking at least one, and sometimes at least 5 or 10 N- or C-terminal amino acids present in naturally occurring forms of Aβ are used in some methods. For example, a fragment lacking 5 amino acids from the C-terminal end of Aβ43 includes the first 38 amino acids from the N-terminal end of Aβ. Unless otherwise indicated, reference to Aβ includes the natural human amino acid sequences indicated above as well as analogs including allelic, species and induced variants. Analogs typically differ from naturally occurring peptides at one, two or a few positions, often by virtue of conservative substitutions. Analogs typically exhibit at least 50% sequence identity with natural peptides, and preferably 60%, 70%, 80% and most preferably 90% sequence identity. Some analogs also include unnatural amino acids or modifications of N or C terminal amino acids at one, two or a few positions. For example, the natural aspartic acid residue at position 1 and/or 7 of Aβ can be replaced with iso-aspartic acid. Examples of unnatural amino acids are D-amino acids, α, α-disubstituted amino acids, N-alkyl amino acids, lactic acid, 4-hydroxyproline, gamma-carboxyglutamate, ε-N,N,N-trimethyllysine, ε -N-acetyllysine, O-phosphoserine, N-acetylserine, N-formylmethionine, 3-methylhistidine, 5-hydroxylysine, ω-N-methylarginine, and

isoaspartic acid. Fragments and analogs can be screened for prophylactic or therapeutic efficacy in transgenic animal models in comparison with untreated or placebo controls as described below.

Aβ, its fragments and analogs can be synthesized by solid phase peptide synthesis or recombinant expression, or can be obtained from natural sources. Automatic peptide synthesizers are commercially available from numerous suppliers, such as Applied Biosystems, Foster City, California. Recombinant expression can be in bacteria, such as E. coli, yeast, insect cells or mammalian cells. Procedures for recombinant expression are described by Sambrook et al., *Molecular Cloning: A Laboratory Manual* (C.S.H.P. Press, NY 2d ed., 1989). Some forms of Aβ peptide are also available commercially (e.g., American Peptides Company, Inc., Sunnyvale, CA and California Peptide Research, Inc. Napa, CA).

Therapeutic peptides also include longer polypeptides that include, for example, an active fragment of Aβ peptide, together with other amino acids. Other amino acids can include those having adjuvant properties or immunostimulant properties and those which serve to increase the stability of the peptide. For example, preferred peptides include fusion proteins comprising a segment of Aβ fused to a heterologous amino acid sequence that induces a helper T-cell response against the heterologous amino acid sequence and thereby a B-cell response against the Aβ segment. Such polypeptides can be screened for prophylactic or therapeutic efficacy in animal models in comparison with untreated or placebo controls as described below. The Aβ peptide, analog, active fragment or other polypeptide can be administered in associated or multimeric form or in dissociated form. Therapeutic peptides also include multimers of monomeric and oligomeric immunogenic peptides. More generally, therapeutic peptides for use in the present invention produce or induce an immune response against an amyloid protein. Antibodies may also bind the soluble form in the periphery to act as a sink and drive Aβ from the brain into the periphery, facilitating the clearance of Aβ from the brain by either modulating Aβ equilibrium from the CNS to the periphery or forming a stable

complex with Aβ in the periphery. Such peptides therefore include, but are not limited to, the component itself and variants thereof, analogs and mimetics of the component that induce and/or cross-react with antibodies to the component, as well as antibodies or T-cells that are specifically reactive with the amyloid peptide. Induction of an immune response can be active, as when an immunogen is administered to induce antibodies or T-cells reactive with the component in a patient, or passive, as when an antibody is administered that itself binds to the amyloid peptide in the patient. Exemplary peptides for inducing or producing an immune response against amyloid protein are described below.

One general class of preferred anti-amyloid peptides consists of peptides that are derived from amyloid proteins. As mentioned above, the hallmark of amyloid diseases is the deposition in an organ or organ of amyloid plaques consisting mainly of fibrils, which, in turn, are composed of characteristic amyloid proteins or peptides. According to the present invention, such an amyloid protein or peptide component is a useful peptide for inducing an anti-amyloid immune response. Table 1 summarizes exemplary fibril-forming proteins that are characteristic of various amyloid diseases. In accordance with this aspect of the present invention, administration to an afflicted or susceptible individual of an immunostimulatory composition which includes the appropriate amyloid protein or peptide, including homologs or fragments thereof, provides therapy or prophylaxis with respect to the amyloid disease.

Other formulations for treating hereditary forms of amyloidosis, discussed above, include compositions that produce immune responses against gelsolin fragments for treatment of hereditary systemic amyloidosis, mutant lysozyme protein (Alys), for treatment of a hereditary neuropathy, mutant alpha chain of fibrinogen (AfibA) for a non-neuropathic form of amyloidosis manifest as renal disease, and mutant cystatin C (Acys) for treatment of a form of hereditary cerebral angiopathy reported in Iceland. In addition, certain hereditary forms of prion disease (e.g., Creutzfeldt-Jacob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome (GSS),

and fatal familial insomnia (FFI)) are characterized by a mutant isoform of prion protein, PrP^{Sc}. This protein can be used in therapeutic compositions for treatment and prevention of deposition of PrP plaques, in accordance with the present invention.

As discussed above, amyloid deposition, either systemic or focal, is also associated with aging. It is a further aspect of the present invention that such deposition can be prevented or treated by administering to susceptible individuals compositions consisting of one or more proteins associated with such aging. Thus, plaques composed of ATTR derived from wild type TTR are frequently found in heart tissue of the elderly. Similarly, certain elderly individuals may develop asymptomatic fibrillar focal deposits of Aβ in their brains; Aβ peptide treatment, as detailed herein may be warranted in such individuals. By way of further example, but not limitation, there are a number of additional, non-hereditary forms of amyloid disease that are candidates for treatment methods of the present invention. β_2 microglobulin fibrillar plaques commonly develop in patients receiving long term hemodialysis or peritoneal dialysis. Such patients may be treated with therapeutic compositions directed to β_2 microglobulin or, more preferably, immunogenic epitopes thereof, in accordance with the present invention.

Hormone-secreting tumors may also contain hormone-derived amyloid plaques, the composition of which are generally characteristic of the particular endocrine organ affected. Thus such fibrils may be made up of polypeptide hormones such as calcitonin (medullary carcinoma of the thyroid), islet amyloid polypeptide (occurring in most patients with Type II diabetes), and atrial natriuretic peptide (isolated atrial amyloidosis). Compositions directed at amyloid deposits which form in the aortic intima in atherosclerosis are also contemplated by the present invention. For example, Westermark, et al. describe a 69 amino acid N-terminal fragment of Apolipoprotein A-I which forms such plaques (Westermark, *et al.* Am. J. Path. 147: 1186-92, 1995); therapeutic compositions of the present

invention include immunological peptides directed to such a fragment, as well as the fragment itself.

The foregoing discussion has focused on amyloid fibril components that may be used as therapeutic peptides in treating or preventing various forms of amyloid disease.

The therapeutic peptide can also be an active fragment or analog of a naturally occurring or mutant fibril peptide or protein that contains an epitope that induces a similar protective or therapeutic immune response on administration to a human. Immunogenic fragments typically have a sequence of at least 3, 5, 6, 10 or 20 contiguous amino acids from a natural peptide. Exemplary Aβ peptide immunogenic fragments include Aβ residues 16-21; residues 13-21; residues 10-21; residues 10-16; residues 25-35 residues 10-22; residues 13-22; residues 16-22; and of Aβ. In some methods, the antibody binds to an epitope comprising a free N - or C-terminal residue of Aβ.

Fragments lacking at least one, and sometimes at least 5 or 10 N- or C-terminal amino acids present in naturally occurring forms of the fibril component are used in some methods. For example, a fragment lacking 5 amino acids from the C-terminal end of Aβ43 includes the first 38 amino acids from the N-terminal end of Aβ. Fragments from the N-terminal half of Aβ are preferred in some methods. Analogs include allelic, species and induced variants. Analogs typically differ from naturally occurring peptides at one or a few positions, often by virtue of conservative substitutions. Analogs typically exhibit at least 80 or 90% sequence identity with natural peptides. Some analogs also include unnatural amino acids or modifications of N or C terminal amino acids. Examples of unnatural amino acids are alpha, alpha-disubstituted amino acids, N-alkyl amino acids, lactic acid, 4-hydroxyproline, (-carboxyglutamate, (-N,N,N-trimethyllysine, (-N-acetyllysine, O-phosphoserine, N-acetylserine, N-formylmethionine, 3-methylhistidine, 5-hydroxylysine, T-N-methylarginine.

Generally, persons skilled in the art will appreciate that fragments and analogs designed in accordance with this aspect of the invention can be screened for cross-reactivity with the naturally occurring fibril components and/or prophylactic or therapeutic efficacy in transgenic animal models as described below. Such fragments or analogs may be used in therapeutic compositions of the present invention, if their immunoreactivity and animal model efficacy is roughly equivalent to or greater than the corresponding parameters measured for the amyloid fibril components.

Such peptides, proteins, or fragments, analogs and other amyloidogenic peptides can be synthesized by solid phase peptide synthesis or recombinant expression, according to standard methods well known in the art, or can be obtained from natural sources. Exemplary fibril compositions, methods of extraction of fibrils, sequences of fibril peptide or protein components are provided by many of the references cited in conjunction with the descriptions of the specific fibril components provided herein. Additionally, other compositions, methods of extracting and determining sequences are known in the art available to persons desiring to make and use such compositions. Automatic peptide synthesizers may be used to make such compositions and are commercially available from numerous manufacturers, such as Applied Biosystems (Perkin Elmer; Foster City, California), and procedures for preparing synthetic peptides are known in the art. Recombinant expression can be in bacteria, such as E. coli, yeast, insect cells or mammalian cells; alternatively, proteins can be produced using cell free *in vitro* translation systems known in the art. Procedures for recombinant expression are described by Sambrook et al., *Molecular Cloning: A Laboratory Manual* (C.S.H.P. Press, NY 2d ed., 1989). Certain peptides and proteins are also available commercially; for example, some forms of Aβ peptide are available from suppliers such as American Peptides Company, Inc., Sunnyvale, California, and California Peptide Research, Inc. Napa, California.

Therapeutic peptides may also be composed of longer polypeptides that include, for example, the active peptide amyloid fragment or analog, together with

other amino acids. For example, Aβ peptide can be present as intact APP protein or a segment thereof, such as the C-100 fragment that begins at the N-terminus of Aβ and continues to the end of APP. Such polypeptides can be screened for prophylactic or therapeutic efficacy in animal models as described below. The Aβ peptide, analog, active fragment or other polypeptide can be administered in associated form (i.e., as an amyloid peptide) or in dissociated form, such as oligomeric, monomeric or soluble form. Therapeutic peptides may also include multimers of monomeric and oligomeric immunogenic peptides or conjugates or carrier proteins, and/or, as mentioned above, may be added to other fibril components, in order to provide a broader range of anti-amyloid plaque activity.

In a further variation, an immunogenic peptide, such as a fragment of Aβ, can be presented by a virus or a bacteria as part of an immunogenic composition. A nucleic acid encoding the immunogenic peptide is incorporated into a genome or episome of the virus or bacteria. Optionally, the nucleic acid is incorporated in such a manner that the immunogenic peptide is expressed as a secreted protein or as a fusion protein with an outer surface protein of a virus or a transmembrane protein of a bacteria so that the peptide is displayed. Viruses or bacteria used in such methods should be nonpathogenic or attenuated. Suitable viruses include adenovirus, HSV, Venezuelan equine encephalitis virus and other alpha viruses, vesicular stomatitis virus, and other rhabdo viruses, vaccinia and fowl pox. Suitable bacteria include Salmonella and Shigella. Fusion of an immunogenic peptide to HBsAg of HBV is particularly suitable. Therapeutic peptides also include peptides and other compounds that do not necessarily have a significant amino acid sequence similarity with Aβ but nevertheless serve as mimetics of Aβ and induce a similar immune response. For example, any peptides and proteins forming β-pleated sheets can be screened for suitability. Anti-idiotypic antibodies against monoclonal antibodies to Aβ or other amyloidogenic peptides can also be used. Such anti-Id antibodies mimic the antigen and generate an immune response to it (see *Essential Immunology* (Roit ed., Blackwell Scientific Publications, Palo Alto, 6th ed.), p. 181). Peptides other

than Aβ peptides should induce an immunogenic response against one or more of the preferred segments of Aβ listed above (e.g., 10-16, 10-21, 13-21, 16-21 and 25-35). Preferably, such peptides induce an immunogenic response that is specifically directed to one of these segments without being directed to other segments of Aβ.

Random libraries of peptides or other compounds can also be screened for suitability. Combinatorial libraries can be produced for many types of compounds that can be synthesized in a step-by-step fashion. Such compounds include polypeptides, beta-turn mimetics, polysaccharides, phospholipids, hormones, prostaglandins, steroids, aromatic compounds, heterocyclic compounds, benzodiazepines, oligomeric N-substituted glycines and oligocarbamates. Large combinatorial libraries of the compounds can be constructed by the encoded synthetic libraries (ESL) method described in Affymax, WO 95/12608, Affymax, WO 93/06121, Columbia University, WO 94/08051, Pharmacopeia, WO 95/35503 and Scripps, WO 95/30642 (each of which is incorporated by reference for all purposes). Peptide libraries can also be generated by phage display methods. See, e.g., Devlin, WO 91/1 8980.

Combinatorial libraries and other compounds are initially screened for suitability by determining their capacity to bind to antibodies or lymphocytes (B or T) known to be specific for Aβ or other amyloidogenic peptides such as ATTR. For example, initial screens can be performed with any polyclonal sera or monoclonal antibody to Aβ or any other amyloidogenic peptide of interest. Compounds identified by such screens are then further analyzed for capacity to induce antibodies or reactive lymphocytes to Aβ or other amyloidogenic peptides. For example, multiple dilutions of sera can be tested on microtiter plates that have been precoated with amyloid peptide, and a standard ELISA can be performed to test for reactive antibodies to Aβ. Compounds can then be tested for prophylactic and therapeutic efficacy in transgenic animals predisposed to an amyloidogenic disease, as described in the Examples. Such animals include, for example, mice bearing a 717 mutation of APP described by Games et al., supra, and mice bearing a 670/671 Swedish

mutation of APP such as described by McConlogue et al., US 5,612,486 and Hsiao et al., Science 274, 99 (1996); Staufenbiel et al., Proc. Natl. Acad. Sci. USA 94, 13287-13292 (1997); Sturchler-Pierrat et al., Proc. Natl. Acad. Sci. USA 94, 13287-13292 (1997); Borchelt et al., Neuron 19, 939-945 (1997)). The same screening approach can be used on other potential peptides such as fragments of Aβ, analogs of Aβ and longer peptides including Aβ, described above.

It is appreciated that immunological responses directed at other amyloid plaque components can also be effective in preventing, retarding or reducing plaque deposition in amyloid diseases. Such components may be minor components of fibrils or associated with fibrils or fibril formation in the plaques, with the caveat that components that are ubiquitous throughout the body, or relatively non-specific to the amyloid deposit, are generally less suitable for use as therapeutic targets.

It is therefore a further discovery of the present invention that peptides that induce an immune response to specific plaque components are useful in treating or preventing progression of amyloid diseases. This section provides background on several exemplary amyloid plaque-associated molecules. Induction of an immune response against any of these molecules, alone or in combination with immunogenic therapeutic compositions against the fibril components described above or against any of the other non-fibril forming components described below, provides an additional anti-amyloid treatment regimen, in accordance with the present invention. Also forming part of the present invention are passive immunization regimens based on such plaque components, as described herein.

By way of example, synuclein is a protein that is structurally similar to apolipoproteins but is found in neuronal cytosol, particularly in the vicinity of presynaptic terminals. There are at least three forms of the protein, termed alpha, beta and gamma synuclein. Recently, it has been shown that alpha and β synuclein are involved in nucleation of amyloid deposits in certain amyloid diseases, particularly Alzheimer's disease. (Clayton, D.F., *et al.*, *TINS* 21(6): 249-255, 1998).

More specifically, a fragment of the NAC domain of alpha and β synuclein (residues 61-95) has been isolated from amyloid plaques in Alzheimer's patients; in fact this fragment comprises about 10% of the plaque that remains insoluble after solubilization with sodium dodecyl sulfate (SDS). (George, J.M., *et al.* Neurosci. News 1: 12-17, 1995). Further, both the full length alpha synuclein and the NAC fragment thereof have been reported to accelerate the aggregation of β-amyloid peptide into insoluble amyloid *in vitro*. (Clayton, *supra*).

Additional components associated with amyloid plaques include non-peptide components. For example, perlecan and perlecan-derived glycosaminoglycans are large heparin sulfate proteoglycans that are present in Aβ-containing amyloid plaques of Alzheimer's disease and other CNS and systemic amyloidoses, including amylin plaques associated with diabetes. These compounds have been shown to enhance Aβ fibril formation. Both the core protein and glycosaminoglycan chains of perlecan have been shown to participate in binding to Aβ. Additional glycosaminoglycans, specifically, dermatan sulfate, chondroitin-4-sulfate, and pentosan polysulfate, are commonly found in amyloid plaques of various types and have also been shown to enhance fibril formation. Dextran sulfate also has this property. This enhancement is significantly reduced when the molecules are de-sulfated. Immunogenic therapeutics directed against the sulfated forms of glycosaminoglycans, including the specific glycosaminoglycans themselves, form an additional embodiment of the present invention, either as a primary or secondary treatment. Production of such molecules, as well as appropriate therapeutic compositions containing such molecules, is within the skill of the ordinary practitioner in the art.

Immunization Procedures

The elicited antibodies present in the host having received the vaccine of the present invention bind at the Aβ(16-21) epitope or other epitopes such as Aβ(10-21), Aβ(13-21). AB (10-22), AB)13-22), AB (16-22) and the N– or C-terminal region of

Aβ and have the ability to prevent or reverse amyloidogenesis. The vaccine of the present invention causes the generation of effective antiamyloidogenic antibodies in the vaccinated host.

A suggested immunization procedure is as follows:

a) prepare a vaccine from an all-D peptide having a sequence substantially the same as that of a naturally occurring β amyloid peptide, namely Aβ (all-L). The all-D peptide includes a full length Aβ (1-42, all-D), a peptide derived from an immunogenic fragment of Aβ (1-42, all-D), and a related peptidomimetic;

b) immunize a host with the vaccine to generate an antibody in the host with a binding site capable of preventing fibrillogenesis, associated cellular toxicity and neurodegeneration.

Suitable pharmaceutically acceptable carriers include, without limitation, any non-immunogenic pharmaceutical adjuvants suitable for oral, parenteral, nasal, mucosal, transdermal, intravascular (IV), intraarterial (IA), intramuscular (IM), and subcutaneous (SC) administration routes, such as phosphate buffer saline (PBS).

The pharmaceutical carriers may contain a vehicle, which carries antigens to antigen-presenting cells. Examples of vehicles are liposomes, immune-stimulating complexes, microfluidized squalene-in-water emulsions, microspheres which may be composed of poly(lactic/glycolic) acid (PLGA). Particulates of defined dimensions (<5 micron) include, without limitation, oil-in-water microemulsion (MF59) and polymeric microparticules.

The carriers of the present invention may also include chemical and genetic adjuvants or immunostimulants to augment immune responses or to increase the antigenicity of immunogens. These adjuvants or immunostimulants exert their immunomodulatory properties through several mechanisms such as lymphoid cell

recruitment, cytokine induction, and the facilitation of DNA entry into cells. Cytokine adjuvants include, without limitation, granulocyte-macrophage colony-stimulating factor, interleukin-12, GM-CSF, synthetic muramyl dipeptide analog or monophosphoryl lipid A. Other chemical adjuvants or immunostimulants include, without limitation, lactic acid bacteria, $Al(OH)_3$, muramyl dipeptides and saponins. Examples of microbial adjuvants include, without limitation, CpG motifs, Freund's, muramyl dipeptide, LPS derivatives, heat shock protein (HSP), lipid A derivative, polysaccharides, cholera toxin, killed Bordetella pertussis and LT (lymphotoxin *E. coli*). Examples of non-microbial adjuvants include, without limitation, aluminum salt, alum, mineral oil, iscoms, liposomes, virosomes, archaeosomes, transfersomes, niosomes, cochleates, proteosomes, calcium phosphate, DDA (dimethyldioctadecylammonium bromide), cytokines, hormones and C3d. Such adjuvants are well understood in the art.

The peptide may be coupled to a carrier that will modulate the half-life of the circulating peptide. This will allow control of the period of protection. The peptide-carrier may also be emulsified in an adjuvant and administrated by a standard immunization route.

The compositions and methods of the present invention can be administered therapeutically or prophylactically to treat diseases associated with amyloid-β fibril formation, aggregation or deposition. The compositions and methods of the invention may also act to ameliorate the course of an amyloid-β related disease using any of the following mechanisms, which are meant to be illustrative and not limiting: by slowing the rate of amyloid-β fibril formation or deposition; by lessening the degree of amyloid-β deposition; by inhibiting, preventing or reducing amyloid-β fibril formation; by inhibiting neurodegeneration or cellular toxicity induced by amyloid-β; by inhibiting amyloid-β induced inflammation; by enhancing the clearance of amyloid-β from the brain; or by reducing the levels of the amyloid-β40 or amyloid-β42 peptides in the brain or the plasma.

In a preferred embodiment, the method is used to treat Alzheimer's disease (e.g. sporadic or familial AD). The method can also be used prophylactically or therapeutically to treat other clinical occurrences of amyloid-β deposition, such as in Down's syndrome individuals and in patients with Cerebral Amyloid Angiopathy, hereditary cerebral amyloid angiopathy, hereditary cerebral hemorrhage or hemorrhagic stroke.

Additionally, abnormal accumulation of APP (β-amyloid precursor protein) and of amyloid-β protein in muscle fibers has been implicated in the pathology of sporadic inclusion body myositis (IBM) (Askanas, V. et al. (1996) *Proc. Natl. Acad. Sci. USA* 93: 1314-1319; Askanas, V. et al. (1995) *Current Opinion in Rheumatology* 7: 486-496). Accordingly, the compounds of the invention can be used prophylactically or therapeutically in the treatment of disorders in which amyloid-β protein is abnormally deposited at non-neurological locations, such as treatment of IBM by delivery of the compounds to muscle fibers.

The vaccine of the present invention will, for the most part, be administered parenterally, such as intravascularly (IV), intraarterially (IA), intramuscularly (IM), subcutaneously (SC), transdermally or the like. In some instances, administration may be oral, nasal, rectal, transdermal or aerosol, where the nature of the vaccine allows for transfer to the vascular system. Usually a single injection will be employed although more than one injection may be used, if desired. Typically the primary immunization will be followed by multiple boosts with an interval of a few weeks, using the same antigen or a further modified antigen if desired. The adjuvants or immunostimulants may also be identical or different if desired. The vaccine may be administered by any convenient means, including syringe, trocar, catheter, or the like. Preferably, the administration will be intravascular, where the site of introduction is not critical to this invention, preferably at a site where there is rapid blood flow, *e.g.*, intravenously, in a peripheral or central vein. Other routes

may find use where the administration is coupled with slow release techniques or a protective matrix.

The use of the vaccine of the present invention in preventing and/or treating Alzheimer's disease and other amyloid related diseases can be validated by raising antibodies against the corresponding all-D peptide and testing them to see if they can effectively inhibit or prevent the fibrillogenesis of the natural amyloid peptide (all-L).

The compounds used to prepare vaccines in accordance with the present invention have the common structure of Formula I:

R'-(P)-R'' (I),

wherein

P is an all-D peptide of a fibril or amyloid protein, *e.g.*, β sheet region, GAG-binding site region, Aβ (1-42, all-D), and macrophage adherence region (10-16, all-D), immunogenic fragments thereof, immunogenic derivatives thereof, protein conjugates thereof, immunogenic peptides thereof, and immunogenic peptidomimetics thereof;

R' is an N-terminal substituent selected from the group consisting of:

- hydrogen;
- lower alkyl groups, *e.g.*, acyclic or cyclic having 1 to 8 carbon atoms, without or with functional groups, *e.g.*, carboxylate, sulfonate and phosphonate;
- aromatic groups;

- heterocyclic groups; and
- acyl groups, *e.g.*, alkylcarbonyl, arylcarbonyl, sulfonyl and phosphonyl groups; and

R″ is a C-terminal substituent, *e.g.*, hydroxy, alkoxy, aryloxy, unsubstituted or substituted amino groups.

R′ and R″ may be identical or different; the alkyl or aryl group of R′ and R″ may further be substituted with organic functionalities selected from the group of halides (F, Cl, Br, and I), hydroxyl, alkoxyl, aryloxyl, hydroxycarbonyl, alkoxylcarbonyl, aryloxycarbonyl, carbamyl, unsubstituted or substituted amino, sulfo or alkyloxysulfonyl, phosphono or alkoxyphosphonyl, and the like.

The term "alkyl" includes saturated aliphatic groups, including straight-chain alkyl groups (*e.g.*, methyl, ethyl, propyl, butyl, pentyl, hexyl, heptyl, and octyl,), branched-chain alkyl groups (*e.g.*, isopropyl, tert-butyl, isobutyl), cycloalkyl (*e.g.*, alicyclic) groups (*e.g.*, cyclopropyl, cyclopentyl, cyclohexyl, cycloheptyl, cyclooctyl), alkyl substituted cycloalkyl groups, and cycloalkyl substituted alkyl groups. "Alkyl" further includes alkyl groups which have oxygen, nitrogen, sulfur or phosphorous atoms replacing one or more hydrocarbon backbone carbon atoms. In certain embodiments, a straight chain or branched chain alkyl has six or fewer carbon atoms in its backbone (e.g., C_1-C_6 for straight chain, C_3-C_6 for branched chain), and more preferably four or fewer. Likewise, preferred cycloalkyls have from three to eight carbon atoms in their ring structure, and more preferably have five or six carbons in the ring structure. "C_1-C_6" includes alkyl groups containing one to six carbon atoms.

The term "alkyl" also includes both "unsubstituted alkyls" and "substituted alkyls", the latter of which refers to alkyl moieties having substituents replacing a hydrogen on one or more carbons of the hydrocarbon backbone. Such substituents can include, for example, alkyl, alkenyl, alkynyl, halogen, hydroxyl, alkylcarbonyloxy, arylcarbonyloxy, alkoxycarbonyloxy, aryloxycarbonyloxy,

carboxylate, alkylcarbonyl, arylcarbonyl, alkoxycarbonyl, aminocarbonyl, alkylaminocarbonyl, dialkylaminocarbonyl, alkylthiocarbonyl, alkoxyl, phosphate, phosphonato, phosphinato, cyano, amino (including alkylamino, dialkylamino, arylamino, diarylamino, and alkylarylamino), acylamino (including alkylcarbonylamino, arylcarbonylamino, carbamoyl and ureido), amidino, imino, sulfhydryl, alkylthio, arylthio, thiocarboxylate, sulfates, alkylsulfinyl, sulfonato, sulfamoyl, sulfonamido, nitro, trifluoromethyl, cyano, azido, heterocyclyl, alkylaryl, or an aromatic or heteroaromatic moiety. Cycloalkyls can be further substituted, *e.g.*, with the substituents described above. An "alkylaryl" or an "aralkyl" moiety is an alkyl substituted with an aryl (*e.g.*, phenylmethyl (benzyl)). "Alkyl" also includes the side chains of natural and unnatural amino acids.

As used herein, "acyl groups" include compounds and moieties which contain the acyl radical (CH_3CO-) or a carbonyl group. "Substituted acyl" includes acyl groups where one or more of the hydrogen atoms are replaced by for example, alkyl groups, alkynyl groups, halogens, hydroxyl, alkylcarbonyloxy, arylcarbonyloxy, alkoxycarbonyloxy, aryloxycarbonyloxy, carboxylate, alkylcarbonyl, arylcarbonyl, alkoxycarbonyl, aminocarbonyl, alkylaminocarbonyl, dialkylaminocarbonyl, alkylthiocarbonyl, alkoxyl, phosphate, phosphonato, phosphinato, phosphonyl, cyano, amino (including alkylamino, dialkylamino, arylamino, diarylamino, and alkylarylamino), acylamino (including alkylcarbonylamino, arylcarbonylamino, carbamoyl and ureido), amidino, imino, sulfhydryl, alkylthio, arylthio, thiocarboxylate, sulfates, alkylsulfinyl, sulfonato, sulfamoyl, sulfonamido, sulfonyl nitro, trifluoromethyl, cyano, azido, heterocyclyl, alkylaryl, or an aromatic or heteroaromatic moiety, i.e. arylcarbonyl.

The term "heterocyclic group" includes closed ring structures, *e.g.*, 3- to 10-, or 4- to 7-membered rings, which include one or more heteroatoms. Heterocyclyl groups can be saturated or unsaturated and include pyrrolidine, oxolane, thiolane, piperidine, piperazine, morpholine, lactones, lactams such as azetidinones and pyrrolidinones, sultams, sultones, and the like. The heterocyclic ring can be

substituted at one or more positions with such substituents as described above, as for example, halogen, hydroxyl, alkylcarbonyloxy, arylcarbonyloxy, alkoxycarbonyloxy, aryloxycarbonyloxy, carboxylate, alkylcarbonyl, alkoxycarbonyl, aminocarbonyl, alkylthiocarbonyl, alkoxyl, phosphate, phosphonato, phosphinato, cyano, amino (including alkyl amino, dialkylamino, arylamino, diarylamino, and alkylarylamino), acylamino (including alkylcarbonylamino, arylcarbonylamino, carbamoyl and ureido), amidino, imino, sulfhydryl, alkylthio, arylthio, thiocarboxylate, sulfates, sulfonato, sulfamoyl, sulfonamido, nitro, trifluoromethyl, cyano, azido, heterocyclyl, or an aromatic or heteroaromatic moiety.

As used herein, the term "aromatic groups" is intended to encompass "aryl" substituents. "Aryl" includes groups with aromaticity, including 5- and 6-membered single-ring aromatic groups that may include from zero to four heteroatoms, as well as multicyclic systems with at least one aromatic ring. Examples of aryl groups include benzene, phenyl, pyrrole, furan, thiophene, thiazole, isothiazole, imidazole, triazole, tetrazole, pyrazole, oxazole, isooxazole, pyridine, pyrazine, pyridazine, and pyrimidine, and the like. Furthermore, the term "aryl" includes multicyclic aryl groups, *e.g.*, tricyclic, bicyclic, e.g., naphthalene, benzoxazole, benzodioxazole, benzothiazole, benzoimidazole, benzothiophene, methylenedioxyphenyl, quinoline, isoquinoline, napthridine, indole, benzofuran, purine, benzofuran, deazapurine, or indolizine. Those aryl groups having heteroatoms in the ring structure may also be referred to as "aryl heterocycles", "heterocycles," "heteroaryls" or "heteroaromatics". The aromatic ring can be substituted at one or more ring positions with such substituents as described above, as for example, halogen, hydroxyl, alkoxy, alkylcarbonyloxy, arylcarbonyloxy, alkoxycarbonyloxy, aryloxycarbonyloxy, carboxylate, alkylcarbonyl, alkylaminocarbonyl, aralkylaminocarbonyl, alkenylaminocarbonyl, alkylcarbonyl, arylcarbonyl, aralkylcarbonyl, alkenylcarbonyl, alkoxycarbonyl, aminocarbonyl, alkylthiocarbonyl, phosphate, phosphonato, phosphinato, cyano, amino (including alkylamino, dialkylamino, arylamino, diarylamino, and alkylarylamino), acylamino (including

alkylcarbonylamino, arylcarbonylamino, carbamoyl and ureido), amidino, imino, sulfhydryl, alkylthio, arylthio, thiocarboxylate, sulfates, alkylsulfinyl, sulfonato, sulfamoyl, sulfonamido, nitro, trifluoromethyl, cyano, azido, heterocyclyl, alkylaryl, or an aromatic or heteroaromatic moiety. Aryl groups can also be fused or bridged with alicyclic or heterocyclic rings which are not aromatic so as to form a multicyclic system (e.g., tetralin, methylenedioxyphenyl).

Where a functional group is an acid, its pharmaceutically acceptable salt or ester is in the scope of this invention. Where a functional group is a base, its pharmaceutically acceptable salt is in the scope of this invention.

In one embodiment, P is a peptide capable of interacting with at least one region of an amyloid protein or fibril.

In another embodiment, the preferred compounds are selected from the full-length peptide, Aβ (1-42, all-D), and its lower homologues consisting of Aβ (1-40, all-D), Aβ (1-35, all-D), Aβ (1-28, all-D), and Aβ (10-21, all-D).

In another embodiment, the preferred compounds are selected from a group of short peptides, *e.g.*, Aβ (1-7, all-D), Aβ (10-16, all-D), Aβ (13-21, all-D), Aβ (16-21, all-D), Aβ (36-42, all-D) AB (10-22 all-D), AB (13-22 all-D) or AB (16-22 all D). The peptides can be shortened further by removing one or more residues from either end or both ends.

The preferred compounds may also be all-D peptides derived from the peptides above by substitution of one or more residues in the sequence with other amino acid residues or non-amino acid fragments, such as an amino alkanesulfonic acid residue.

In a further embodiment, the preferred compounds may be coupled with a carrier that will modulate the biodistribution, immunogenic property and the half-life of the compounds.

The following are exemplary compounds for preparing vaccines for preventing or treating Alzheimer's disease and other amyloid related diseases:

SEQ ID NO: 1	Aβ (1-42, all-D) DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMV GGVVIA
SEQ ID NO: 2	Aβ (1-40, all-D) DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMV GGVV
SEQ ID NO: 3	Aβ (1-35, all-D) DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLM
SEQ ID NO: 4	Aβ (1-28, all-D) DAEFRHDSGYEVHHQKLVFFAEDVGSNK
SEQ ID NO: 5	Aβ (1-7, all-D) DAEFRHD
SEQ ID NO: 6	Aβ (10-16, all-D) YEVHHQK
SEQ ID NO: 7	Aβ (16-21, all-D) KLVFFA
SEQ ID NO: 8	Aβ (10-21, all-D) YEVHHQKLVFFA
SEQ ID NO: 9	Aβ (13-21, all-D) HHQKLVFFA
SEQ ID NO: 10	Aβ (36-42, all-D) VGGVVIA
SEQ ID NO: 11	Lys-Ile-Val-Phe-Phe-Ala (all-D)

SEQ ID NO: 12 Lys-Lys-Leu-Val-Phe-Phe-Ala (all-D)

SEQ ID NO: 13 Lys-Phe-Val-Phe-Phe-Ala (all-D)

SEQ ID NO: 14 Ala-Phe-Phe-Val-Leu-Lys (all-D)

SEQ ID NO: 15 Lys-Leu-Val-Phe (all-D)

SEQ ID NO: 16 Lys-Ala-Val-Phe-Phe-Ala (all-D)

SEQ ID NO: 17 Lys-Leu-Val-Phe-Phe (all-D)

SEQ ID NO: 18 Lys-Leu-Val-Phe-Phe-Ala-NH_2 (all-D)

SEQ ID NO: 19 Lys-Ile-Val-Phe-Phe-Ala-NH_2 (all-D)

SEQ ID NO: 20 Lys-Leu-Val-Phe-Phe-Ala-NH_2 (all-D)

SEQ ID NO: 21 Lys-Phe-Val-Phe-Phe-Ala-NH_2 (all-D)

SEQ ID NO: 22 Ala-Phe-Phe-Val-Leu-Lys-NH_2 (all-D)

SEQ ID NO: 23 Lys-Leu-Val-Phe-NH_2 (all-D)

SEQ ID NO: 24 Lys-Ala-Val-Phe-Phe-Ala-NH_2 (all-D)

SEQ ID NO: 25 Lys-Leu-Val-Phe-Phe-NH_2 (all-D)

SEQ ID NO: 26 Lys-Val-Val-Phe-Phe-Ala-NH_2 (all-D)

SEQ ID NO: 27 Lys-Leu-Val-Phe-Phe-Ala-Glu (all-D)

SEQ ID NO: 28 Lys-Leu-Val-Phe-Phe-Ala-Glu-NH_2 (all-D)

SEQ ID NO: 29 His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu (all-D)

SEQ ID NO: 30 Asp-Asp-Asp (all-D)

SEQ ID NO: 31 Lys-Val-Asp-Asp-Gln-Asp (all-D)

SEQ ID NO: 32 His-His-Gln-Lys (all-D)

SEQ ID NO: 33 Phe-Phe-NH-$CH_2CH_2SO_3H$ (all-D)

SEQ ID NO: 34 Phe-Phe-NH-$CH_2CH_2CH_2SO_3H$ (all-D)

SEQ ID NO: 35 Phe-Phe-NH-$CH_2CH_2CH_2CH_2SO_3H$ (all-D)

SEQ ID NO: 36 Phe-Tyr-NH-$CH_2CH_2SO_3H$ (all-D)

SEQ ID NO: 37 Phe-Tyr-NH-$CH_2CH_2CH_2SO_3H$ (all-D)

SEQ ID NO: 38 Phe-Tyr-NH-$CH_2CH_2CH_2CH_2SO_3H$ (all-D)

SEQ ID NO: 39 $HO_3SCH_2CH_2$-Phe-Phe (all-D)

SEQ ID NO: 40 $HO_3SCH_2CH_2CH_2$-Phe-Phe (all-D)

SEQ ID NO: 41 $HO_3SCH_2CH_2CH_2CH_2$-Phe-Phe (all-D)

SEQ ID NO: 42 $HO_3SCH_2CH_2$-Phe-Tyr (all-D)

SEQ ID NO: 43 $HO_3SCH_2CH_2CH_2$-Phe-Tyr (all-D)

SEQ ID NO: 44 $HO_3SCH_2CH_2CH_2CH_2$-Phe-Tyr (all-D)

SEQ ID NO: 45 $HO_3SCH_2CH_2$-Leu-Val-Phe-Phe-Ala (all-D)

SEQ ID NO: 46 $HO_3SCH_2CH_2CH_2$-Leu-Val-Phe-Phe-Ala (all-D)

SEQ ID NO: 47 $HO_3SCH_2CH_2CH_2CH_2$-Leu-Val-Phe-Phe-Ala (all-D)

SEQ ID NO: 48 Leu-Val-Phe-Phe-Ala-NH-$CH_2CH_2SO_3H$ (all-D)

SEQ ID NO: 49 Leu-Val-Phe-Phe-Ala-NH-$CH_2CH_2CH_2SO_3H$ (all-D)

SEQ ID NO: 50 Leu-Val-Phe-Phe-Ala-NH-$CH_2CH_2CH_2CH_2SO_3H$ (all-D)

SEQ ID NO: 51 Aβ ([L] 10-15 [D] 16-21)

Y[L]-E[L]-V[L]-H[L]-H[L]-Q[L]-K[D]-L[D]-V[D]-F[D]-F[D]-A[D]

SEQ ID NO: 52 Aβ ([D] 16-21 [L] 22-28)

K[D]-L[D]-V[D]-F[D]-F[D]-A[D]-E[L]-D[L]-V[L]-G[L]-S[L]-N[L]-K[L]

SEQ ID NO: 53 His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val (all-D)

SEQ ID NO: 54 Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys (all-D)

SEQ ID NO: 55
Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala (all-D)

The compounds listed above may be modified by removing or inserting one or more amino acid residues, or by substituting one or more amino acid residues with other amino acid or non-amino acid moieties or fragments. .

The following are exemplary compounds derived from compound 18 (all-D KLVFFA-NH_2; SEQ ID NO: 18) by substituting one or two amino acid residue(s) with other amino acids.

SEQ ID NO: 56 Lys-Leu-Val-Trp-Phe-Ala-NH_2(all-D)

SEQ ID NO: 57 Lys-Leu-Val-Phe-Trp-Ala- NH_2 (all-D)

SEQ ID NO: 58 Lys-Leu-Val-Trp-Trp-Ala- NH_2 (all-D)

SEQ ID NO: 59 Lys-Leu-Val-Tyr-Phe-Ala- NH_2 (all-D)

SEQ ID NO: 60 Lys-Leu-Val-Phe-Tyr-Ala- NH_2 (all-D)

SEQ ID NO: 61 Lys-Leu-Val-Tyr-Tyr-Ala- NH_2 (all-D)

SEQ ID NO: 62 Lys-Leu-Val-Thi-Phe-Ala- NH_2 (all-D)

SEQ ID NO: 63 Lys-Leu-Val-Phe-Thi-Ala- NH_2 (all-D)

SEQ ID NO: 64 Lys-Leu-Val-Thi-Thi-Ala- NH_2 (all-D)

SEQ ID NO: 65 Lys-Leu-Val-Cha-Phe-Ala- NH_2 (all-D)

SEQ ID NO: 66 Lys-Leu-Val-Phe-Cha-Ala- NH_2 (all-D)

SEQ ID NO: 67 Lys-Leu-Val-Cha-Cha-Ala- NH_2 (all-D)

SEQ ID NO: 68 Lys-Leu-Val-Pgly-Phe-Ala- NH_2 (all-D)

SEQ ID NO: 69 Lys-Leu-Val-Phe-Pgly-Ala- NH_2 (all-D)

SEQ ID NO: 70 Lys-Leu-Val-Pgly-Pgly-Ala- NH_2 (all-D).

For the above compounds, the terms Thi, Cha and Pgly are intended to mean thienylalanine, cyclohexylalanine and phenylglycine, respectively.

Rabbits were immunized with all-D or all-L KLVFFA. Results of the antibody titers obtained are shown in FIG. 7. As seen in FIG. 7, the vaccine of the present invention causes production of antibodies.

In another experiment, adult TgCRND8 mice were immunized with all-D or all-L KLH-amyloid-β fragment ([L]-amyloid-β 13-21 and [D] amyloid-β 13-21). Results of the antibody titers is shown in Table 2.

Table 2

KLH-amyloid-β fragment	Mean Plasma Antibody Titer (inverse) in TgCRND8
[L] amyloid-β 13-21	<100
[D] amyloid-β 13-21	11,000

The present invention encompasses various types of immune responses triggered using the vaccine of the present invention, *e.g.*, amyloid therapies using the vaccine approach.

In accordance with the present invention, there is also provided a vaccine which triggers a preferential TH-2 response or a TH-1 response, according to the type of immunization used. By inducing a TH-2 response, anti-inflammatory cytokine production such as IL-4, Il-10 and TGF-β, as well as the production of IgG 1 and IgG 2b antibody classes, are favored. Such type of response would be preferred, as a major inflammatory response in the brain of the patients with AD would be avoided. On the other hand, with a preferred TH-1 response, a pro-inflammatory response with a production of inflammatory cytokines such as IL-1, IL-6, TNF and IFN gamma would be favored. This type of response would more likely trigger activation of the macrophage population. These macrophages would then phagocytose any particulate deposits (such as plaques) via a complement-activated process as well as via an antibody-mediated process. This approach would be beneficial to clear already organized senile plaques and prevent the formation of new fibrillary deposits. However this response would also be accompanied by an inflammatory response which would be detrimental to the host.

Both approaches (*i.e.* TH-1 and TH-2) are of value. The antigen used could be the peptides which contain regions responsible for cellular adherence, *i.e.*, region 10-16, regions responsible for the GAG binding site, *i.e.*, 13-16, regions responsible for the β sheet, *i.e.* 16-21, or regions specific for the C-terminus, *i.e.* 40-42. These peptides could be presented in such a way that either a preferential TH-1 or TH-2 response is obtained, depending on the type of adjuvant used, or depending on the route of administration of the vaccine. For example, a mucosal immunization via nasal administration is possible, since it is known that such a route of administration would favor a TH-2 response.

The present invention will be more readily understood by referring to the following examples, which are given to illustrate the invention rather than to limit its scope.

EXAMPLE I

An *in vitro* validation procedure to test the effectiveness of all-D peptide vaccines derived from fibrillogenic amyloid proteins was performed in rabbits or mice to demonstrate that antibodies can be raised against Aβ 16-21 (all-D) (see FIG. 7). The antibodies produced were tested to prove that they effectively prevent the fibrillogenesis of natural Aβ (1-40, all-L) *in vitro*. Standard assays for fibrillogenesis were used to evaluate activity, such as those based on Thioflavine T, circular dichroism and solubility.

This approach was also used to establish which areas of the Aβ peptide are most effective when used in the form of all-D peptides to prepare antifibrillogenic vaccines. The experiments were performed as follows:

a) rabbits or mice were immunized with a series of overlapping all-D peptides generated from the Aβ (1-42) sequence, *e.g.*, Aβ (16-21), Aβ (10-21), Aβ (13-21), etc.

b) antisera were prepared from the immunized rabbits or mice.

c) these antisera were tested to see which parts of the Aβ sequence produce antisera that most effectively prevent fibrillogenesis in the standard assays for fibrillogenesis mentioned above.

EXAMPLE II

Effect of Antibodies Against D- and L-Aβ (16-21) Peptide Vaccine on Fibrillogenesis

A validation procedure to test anti-fibrillogenic activity of antibodies raised against D- and L- Aβ (16-21) peptide was performed.

Rabbits were immunized with D- or L-Aβ (16-21) peptide. Antibodies raised were tested for their antifibrillogenic activities by ThT assay and by electron microscopy (EM).

Antibodies raised against the D- and L- forms of KLVFFA were capable of blocking the fibrillogenesis process as seen either by the Thioflavin T assay (ThT) (FIGs. 2 and 3) and by EM (FIGs. 4A to 4C). In the ThT assay, fibril formation is monitored by the increase in fluorescence with time. As seen in the Figures, the antibodies were capable of inhibiting such an increase in fluorescence, proving that these antibodies were inhibiting fibrillogenesis.

As can be seen in these figures (FIGs. 2 to 4), antibodies raised against the D-peptide have a better anti-fibrillogenic activity than anti-L antibodies.

These results were also confirmed by EM (FIGs. 4A to 4C) where both anti-D and anti-L KLVFFA peptide blocked the fibril formation when compared to control (FIG. 4A). Moreover, again the anti-D peptide has a greater anti-fibrillogenic activity (FIG. 4B) than the anti-L peptide (FIG. 4C). This goes along with the ThT assay where the decrease in fluorescence was greater with the anti-D peptide antibody than with the anti-L peptide antibody.

EXAMPLE III

Antibody Binding Assay

Brain sections were stained with antibodies raised against KLVFFA peptide (D and L forms). As seen in FIGs 5A to 5D and 6A to 6D, the antibodies were not capable of binding to aggregated (ThioS positive) Aβ. It can be seen from both sets of figures, which were stained for both plaques (using ThioS) and Aβ peptide (using the antibodies raised) that the antibodies recognize Aβ at the surface of the cells but are not capable of binding to plaques. These results show that the anti-KLVFFA peptide antibody does not bind to aggregated Aβ. These results clearly prove that the antibody recognizes only the non-aggregated form and blocks fibrillogenesis. By having such activity, the vaccine of the present invention 1) prevents Aβ from organizing itself into a fibril and 2) prevents an inflammatory response being triggered by such an antibody binding to an insoluble form, since the antibody is not able to bind to aggregated Aβ. Furthermore, by maintaining Aβ in a soluble form, such antibody may favor the clearance of Aβ from the brain prior to its organization as a fibril. Such antibody would also maintain a beneficial equilibrium between the concentration of Aβ in the brain vs. the plasma.

EXAMPLE IV

Effect of Antibodies Against D- and L- Aβ (13-21-KLH) Peptide Vaccine on Amyloid-β Accumulation in the Brain and Plasma

Adult TgCRND8 mice (which carry a transgene expressing the human Amyloid Precursor Protein (hAPP)) were immunized with D or L peptide at 9 weeks of age, and every two weeks thereafter (a total of 5 injections). Animals were sacrificed 10-14 days after the last boost and the accumulation of amyloid-β in the brain and plasma of the mice was determined. Brains were homogenized as follows:

First, brains were frozen and the frozen tissue was weighed. Tissue was then homogenized in 4 volumes of 50 mM Tris-HCl pH 8.0 containing 1x protease inhibitor cocktail (Calbiochem #539131) using a Wheaton Overhead Stirrer, speed 3.6. Samples were vortexed and spun in an Eppendorf centrifuge at 16,000x g for 20 min at 4°C. 150 µL of the supernatant was added to 250 µL 8 M guanidium/50 mM Tris-HCl pH8.0. The mixture was vortexed and stored at –80°C. The pellet was thawed and resuspended by vortexing in 7 volumes of 5 M guanidium/50 mM Tris pH8.0 and stored at –80°C. The supernatant and pellet were thawed and sonicated for 15 min at 80°C in an Elma Ultrasonic bath, then frozen on dry ice for 15 min, then thawed and sonicated in an Elma Ultrasonic bath at 80°C for 10 min. Samples were then spun at 16,000x g for 20 min at 4°C, and the supernatant was kept for amyloid-β quantification. Amyloid-β40 and amyloid-β42 were quantified using a human β Amyloid ELISA kit from Biosource International (catalog #88-344).

Specific plasma antibody titers were determined using a standard ELISA procedure (shown in Table 2, *supra*). The data in table 2 indicates that immunization with the [D] 13-21 peptide was much more effective at inducing an immune response than immunization with the [L] 13-21 peptide.

The results also showed a reduction in both soluble and fibrillar (insoluble) Aβ40 level (FIGs. 8A and 8B), a reduction in both soluble and fibrillar Aβ42 level (FIG. 9) and a reduction in total Aβ level (FIGs. 10A and 10B) in the brain of TgCRND8 mice after immunization with [D]13-21-KLH. The results indicate that immunization with D peptides was effective at reducing Aβ levels. Also, D peptides were much more potent than L peptides, as only immunization with D peptides reduced Aβ levels, and immunization with L peptides did not reduce Aβ levels.

EXAMPLE V

Passive Immunization Against Amyloid-beta

Humanized antibodies are raised and obtained from transgenic animals such as Xenomouse® (Abgenix, Freemont, CA), by exposing the animal to an antigenic amount of an all-D Amyloid-β (10-22). An antigenic amount ranges from 10ng to 10mg of the all-D Amyloid-β peptide. The immune response can be enhanced by the use of adjuvants as described, thus permitting lower concentrations of the immunogen to be used. The serum antibody levels are monitored through ELISA or other methods of titering an immune response. The antibodies may be directly recovered from the serum of the animal, or B-cells expressing such antibodies may be recovered from an immunized animal for creation of hybridoma lines. The humanized antibodies are purified from serum, ascites or cell culture medium by Protein G chromatography or similar methods of antibody purification.

The antibodies, raised directly in the second or donor mammal or indirectly by creation of a hybridoma line derived from an immune cell from the donor mammal, can be used to immunize a first or subject mammal suffering from an amyloid related disease or disorder, or to prevent an amyloid related disease or disorder. The first mammal is provided with a therapeutically or prophylactically effective dose of the anti-Aβ antibodies. Antibody fragments or primed immune cells such as B-cells, T-cells, or primed antigen presenting cells can also be obtained from a donor, preferably a human donor and more preferably a syngeneic human donor. A therapeutically effective dose is identified as one which decreases the formation or mass of fibrillar plaques in the first mammal, by at least 5 to 50% or greater, as measured by the techniques and assays described. A 100% or greater reduction in plaque formation or mass is considered a therapeutic endpoint.

Physicians or other medical professionals can monitor dosing regimens to achieve a therapeutic effect or endpoint.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

What is claimed is:

1. A method for preventing and/or treating an amyloid-related disease in a subject, comprising: administering to the subject an antigenic amount of an all-D amyloid-β peptide, wherein said all-D amyloid-β peptide induces an immune response by said subject against said amyloid-β peptide, and wherein said all-D amyloid-β peptide has an amino acid sequence that is at least 80% identical to a sequence selected from the group consisting of SEQ ID NOS: 1-70.

2. The method of claim 1, wherein said all-D peptide interacts with at least one region of an amyloid protein, said region being selected from the group consisting of: an N- or C-terminal region, β sheet region, GAG-binding site region, macrophage adherence region, immunogenic fragments thereof, protein conjugates thereof, immunogenic derivative peptides thereof, immunogenic peptides thereof, and immunogenic peptidomimetics thereof.

3. The method of claim 2, wherein said all-D peptide further comprises:

(a) an N-terminal substituent selected from the group consisting of:

hydrogen;

lower alkyl group consisting of acyclic or cyclic having 1 to 8 carbon atoms;

aromatic group;

heterocyclic group; and

acyl group; and

(b) a C-terminal substituent selected from the group consisting of hydroxy, alkoxy, aryloxy, unsubstituted and substituted amino groups.

4. The method of claim 3, wherein said alkyl or aromatic group is further substituted with a group selected from the group consisting of halide, hydroxyl, alkoxyl, aryloxyl, hydroxycarbonyl, alkoxylcarbonyl, aryloxycarbonyl, carbamyl, unsubstituted amino, substituted amino, sulfo, alkyloxysulfonyl, phosphono and alkoxyphosphonyl groups.

5. The method of claim 1, wherein said all-D peptide further comprises an acid functional group, or a pharmaceutically acceptable salt or ester form thereof.

6. The method of claim 1, wherein said all-D peptide further comprises a base functional group, or a pharmaceutically acceptable salt form thereof.

7. The method of claim 1, wherein said all-D peptide elicits the clearance of soluble amyloid-β, thereby preventing or reducing amyloid-induced cellular toxicity, neurodegeneration, or reducing or inhibiting the formation of plaques.

8. A method for preventing and/or treating an amyloid-related disease in a first mammal in need thereof, comprising administering an antigenic amount of an all-D amyloid-β peptide to a second mammal, wherein said all-D amyloid-β peptide induces an immune response against the amyloid-β peptide in said second mammal, recovering the antibodies or immune cells from said second mammal, and providing a therapeutically or prophylactically effective dose of the antibodies or immune cells to said first mammal.

9. The method of claim 8 wherein said all-D amyloid-β peptide has an amino acid sequence that is at least 80% identical to a sequence selected from the group consisting of SEQ ID NOS: 1-70.

10. The method of claim 8, wherein said all-D peptide interacts with at least one region of an amyloid protein, said region being selected from the group consisting of: C-terminal region, β sheet region, GAG-binding site region, macrophage adherence region, immunogenic fragments thereof, protein conjugates thereof, immunogenic derivative peptides thereof, immunogenic peptides thereof, and immunogenic peptidomimetics thereof.

11. The method of claim 8, wherein said all-D peptide further comprises:

(a) an N-terminal substituent selected from the group consisting of:

hydrogen;

lower alkyl group consisting of acyclic or cyclic having 1 to 8 carbon atoms;

aromatic group;

heterocyclic group; and

acyl group; and

(b) a C-terminal substituent selected from the group consisting of hydroxy, alkoxy, aryloxy, unsubstituted and substituted amino groups.

12. The method of claim 8, wherein said alkyl or aromatic group is further substituted with a group selected from the group consisting of halide, hydroxyl, alkoxyl, aryloxyl, hydroxycarbonyl, alkoxylcarbonyl, aryloxycarbonyl, carbamyl, unsubstituted amino, substituted amino, sulfo, alkyloxysulfonyl, phosphono and alkoxyphosphonyl groups.

13. The method of claim 8, wherein said all-D peptide further comprises an acid functional group, or a pharmaceutically acceptable salt or ester form thereof.

14. The method of claim 8, wherein said all-D peptide further comprises a base functional group, or a pharmaceutically acceptable salt form thereof.

15. The method of claim 8, wherein said all-D peptide elicits the clearance of soluble amyloid-β, thereby preventing or reducing amyloid-induced cellular toxicity, neurodegeneration, or reducing or inhibiting the formation of plaques.

16. A method for altering serum levels of amyloid-β in a mammal comprising administering to the subject an antigenic amount of an all-D amyloid-β peptide, wherein said all-D amyloid-β peptide induces an immune response by said subject against said amyloid-β peptide, and wherein said all-D amyloid-β peptide has an amino acid sequence that is at least 80% identical to a sequence selected from the group consisting of SEQ ID NOS: 1-70.

17. The method of claim 16, wherein altering serum levels of amyloid-β in a mammal favors the clearance of soluble amyloid-β or fibril amyloid-β from the mammal.

18. The method of claim 17, wherein the clearance of soluble amyloid-β or fibril amyloid-β from the mammal thereby reduces or inhibits the formation of plaques in the brain of said mammal.

19. The method of claim 16, wherein said all-D peptide further comprises:

(a) an N-terminal substituent selected from the group consisting of:

hydrogen;

lower alkyl group consisting of acyclic or cyclic having 1 to 8 carbon atoms;

aromatic group;

heterocyclic group; and

acyl group; and

(b) a C-terminal substituent selected from the group consisting of hydroxy, alkoxy, aryloxy, unsubstituted and substituted amino groups.

20. The method of claim 16, wherein said alkyl or aromatic group is further substituted with a group selected from the group consisting of halide, hydroxyl, alkoxyl, aryloxyl, hydroxycarbonyl, alkoxylcarbonyl, aryloxycarbonyl, carbamyl, unsubstituted amino, substituted amino, sulfo, alkyloxysulfonyl, phosphono and alkoxyphosphonyl groups.

21. The method of claim 16, wherein said all-D peptide further comprises an acid functional group, or a pharmaceutically acceptable salt or ester form thereof.

22. The method of claim 16, wherein said all-D peptide further comprises a base functional group, or a pharmaceutically acceptable salt form thereof.

23. The method of claim 16, wherein said all-D peptide elicits the clearance of soluble amyloid-β, thereby preventing or reducing amyloid-induced cellular toxicity or neurodegeneration.

24. The method of claim 1, wherein said amyloid-related disease is a disorder

comprising excessive amyloid-β peptides, and inducing said immune response by said subject against said amyloid-β peptides thereby reduces the excessive levels of said amyloid-β peptides, thereby treating or preventing said amyloid-related disease.

25. The method of claim 24, wherein said amyloid-related disease is Alzheimer's disease.

26. The method of claim 24, wherein said amyloid-related disease is cerebral amyloid angiopathy.

27. The method of claim 8, wherein said amyloid-related disease is a disorder comprising excessive amyloid-β peptides, and inducing said immune response by said subject against said amyloid-β peptides thereby reduces the excessive levels of said amyloid-β peptides, thereby treating or preventing said amyloid-related disease.

28. The method of claim 27, wherein said amyloid-related disease is Alzheimer's disease.

29. The method of claim 27, wherein said amyloid-related disease is cerebral amyloid angiopathy.

30. The method of claim 16, wherein said amyloid-related disease is a disorder comprising excessive amyloid-β peptides, and inducing said immune response by said subject against said amyloid-β peptides thereby reduces the excessive levels of said amyloid-β peptides, thereby treating or preventing said amyloid-related disease.

31. The method of claim 30, wherein said amyloid-related disease is Alzheimer's

disease.

32. The method of claim 30, wherein said amyloid-related disease is cerebral amyloid angiopathy.

5

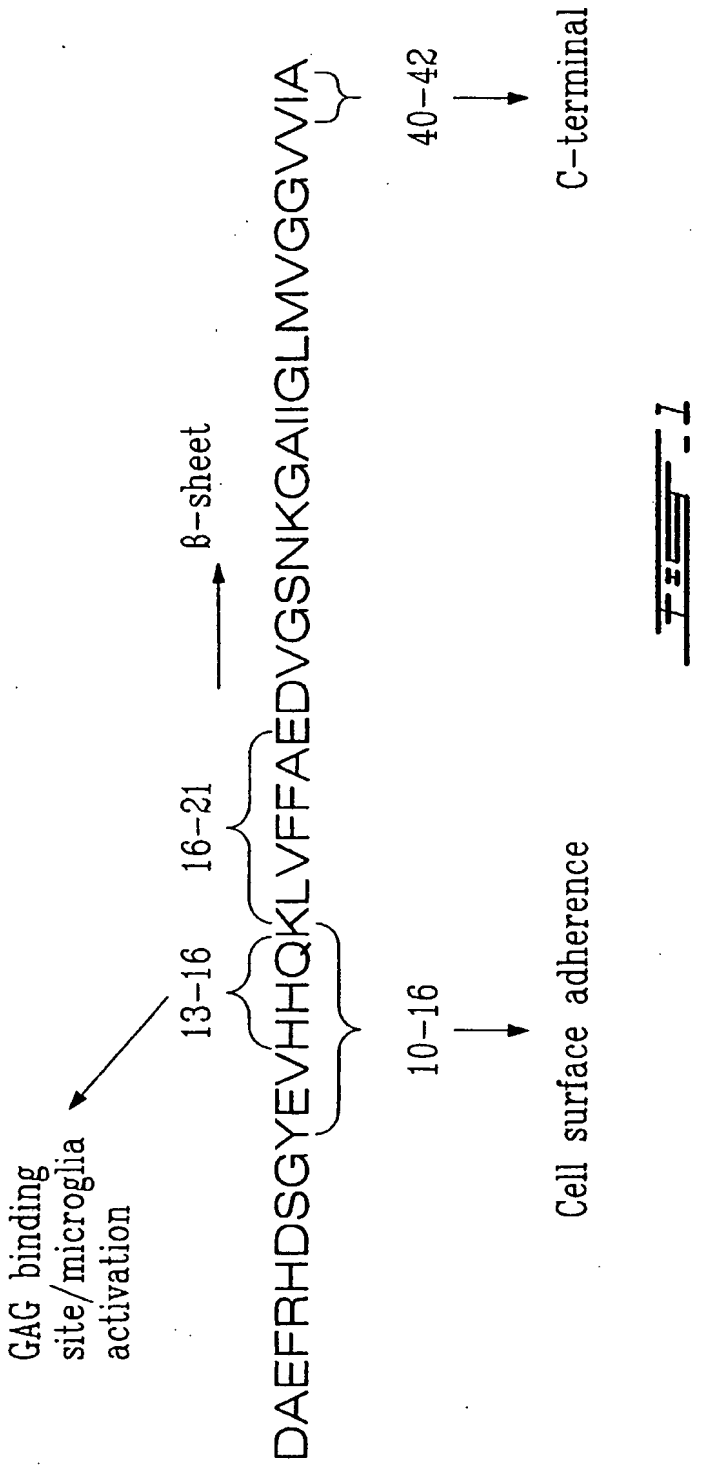
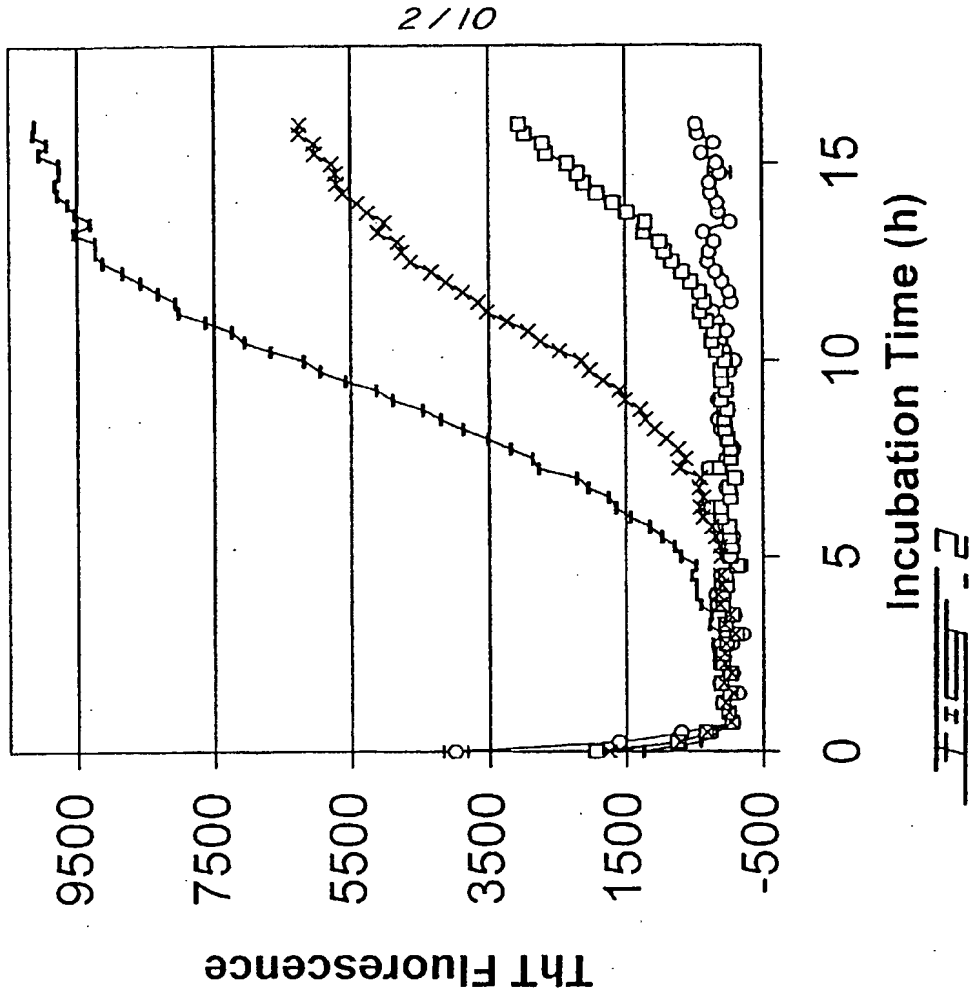

DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
GAG binding site/microglia activation
13-16
16-21
β-sheet
10-16
Cell surface adherence
40-42
C-terminal

Fig. 1


ThT Fluorescence
9500
7500
5500
3500
1500
-500
0
5
10
15
Incubation Time (h)
Anti [D]-KLVFFA
No antibodies
Anti [L]-KLVFFA
Control antibody

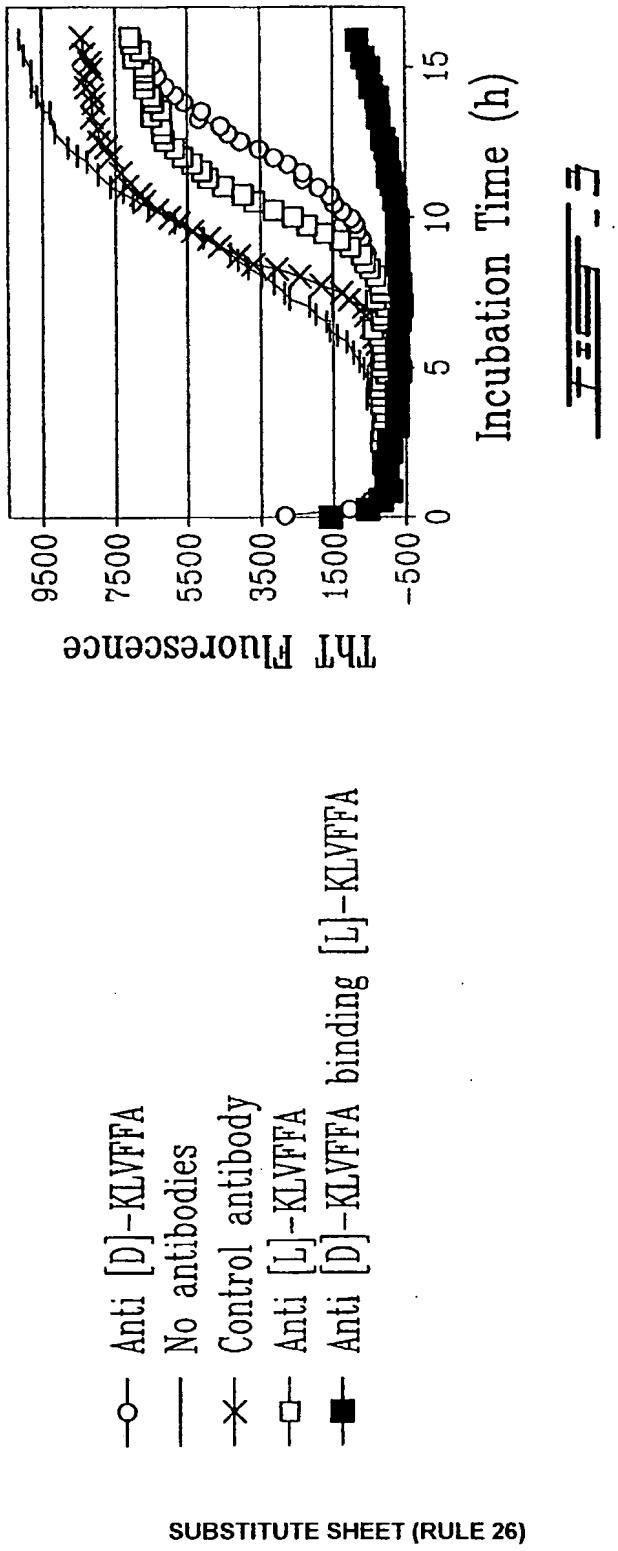

ThT Fluorescence
9500
7500
5500
3500
1500
-500
0
5
10
15
Incubation Time (h)
Anti [D]-KLVFFA
No antibodies
Control antibody
Anti [L]-KLVFFA
Anti [D]-KLVFFA binding [L]-KLVFFA

Fig. 3

Fig. 4C

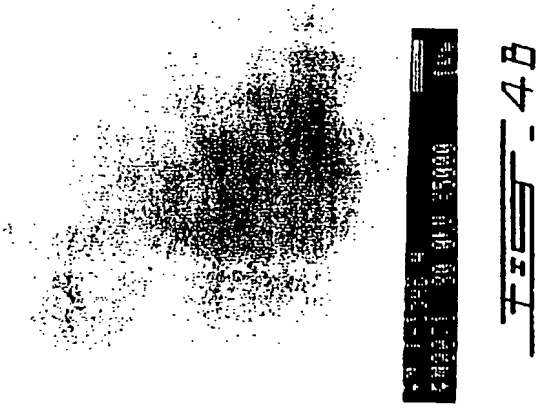


Fig. 4B

Fig. 4A

IMMUNOHISTOCHEMISTRY FOR A 151

FIG - 5A

HISTOCHEMISTRY FOR THIOFLAVIN S

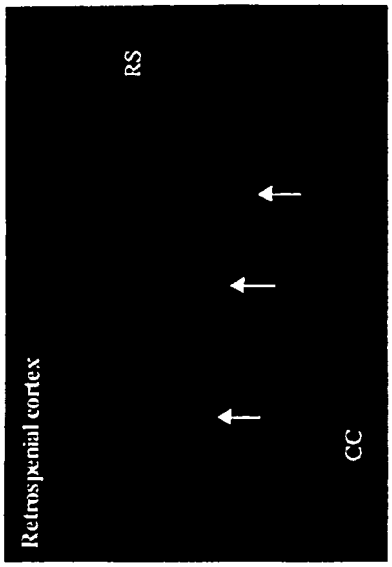


FIG - 5B

IMMUNOHISTOCHEMISTRY FOR A 151

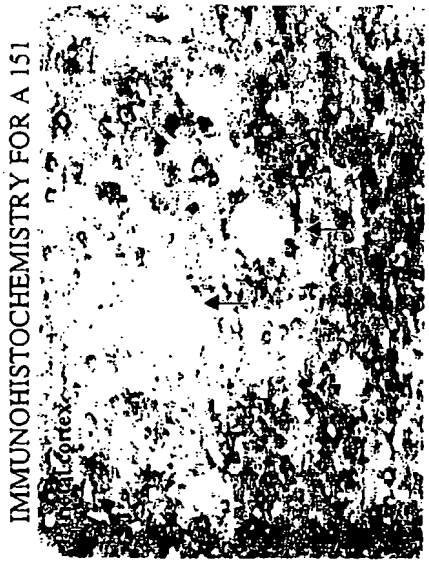


FIG - 5C

HISTOCHEMISTRY FOR THIOFLAVIN S

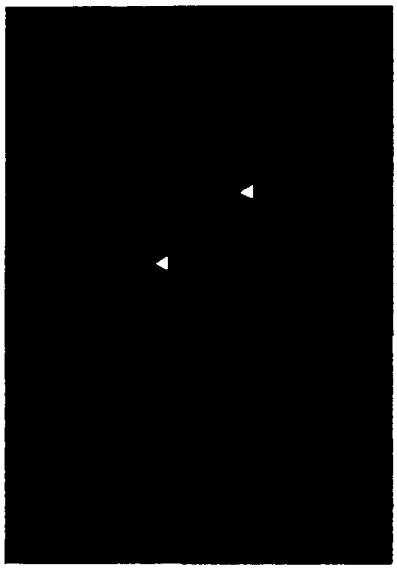


FIG - 5D

HISTOCHEMISTRY FOR THIOFLAVIN S

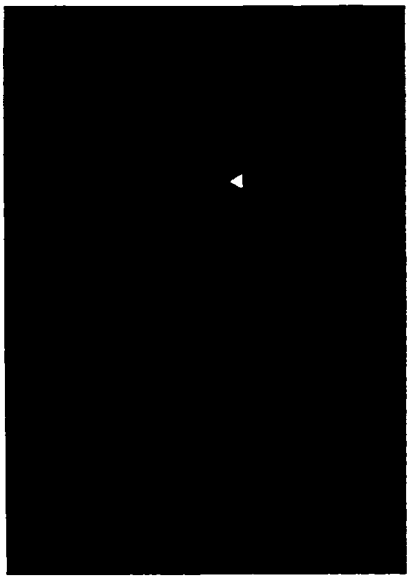


Fig - 6B

HISTOCHEMISTRY FOR THIOFLAVIN S

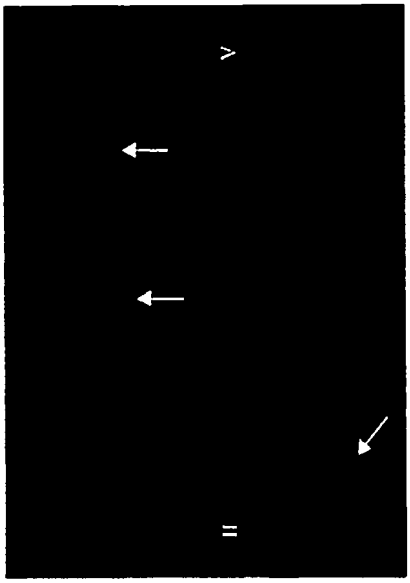


Fig - 6D

IMMUNOHISTOCHEMISTRY FOR C151

Parietal cortex

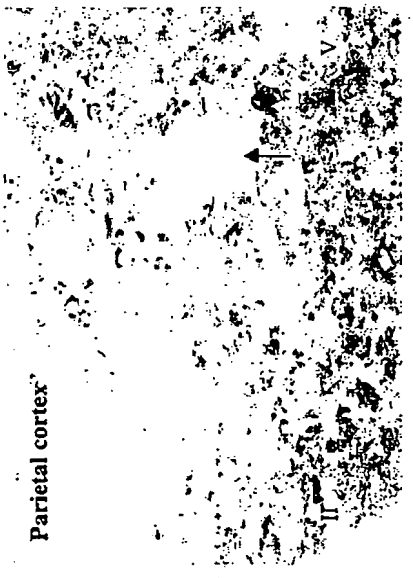


Fig - 6A

IMMUNOHISTOCHEMISTRY FOR C151

Entorhinal cortex

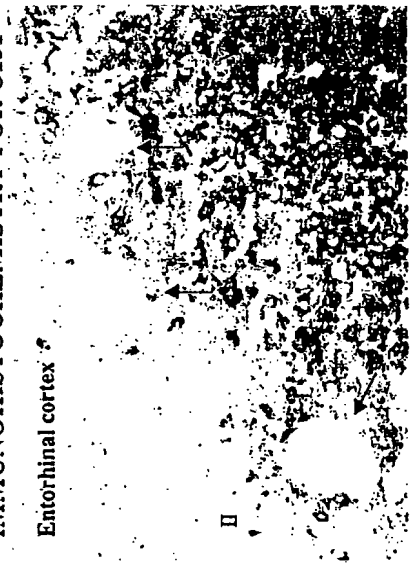


Fig - 6C

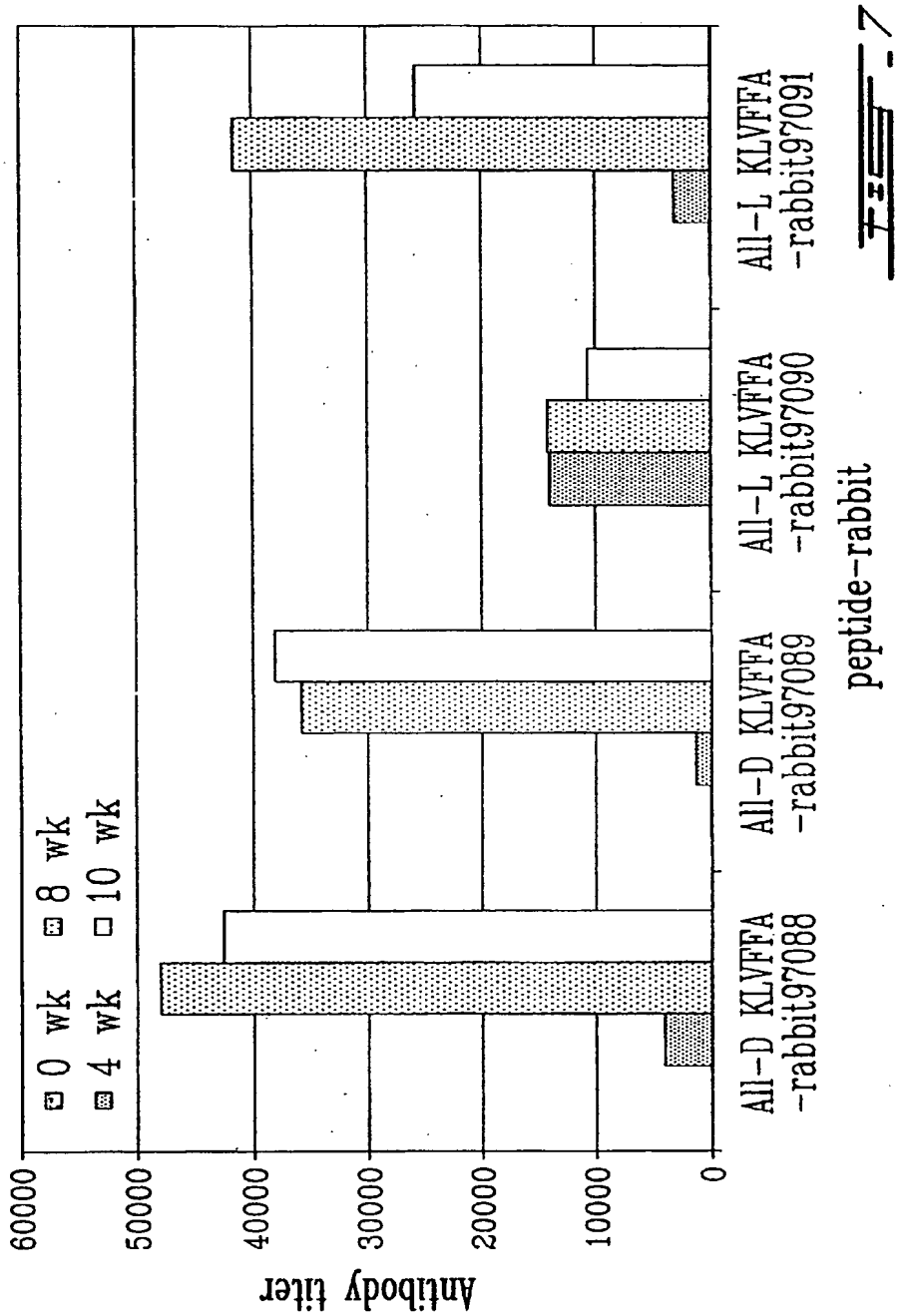

Antibody titer
60000
50000
40000
30000
20000
10000
0
0 wk
8 wk
4 wk
10 wk
All-D KLVFFA -rabbit97088
All-D KLVFFA -rabbit97089
All-L KLVFFA -rabbit97090
All-L KLVFFA -rabbit97091
peptide-rabbit

Fig - 7

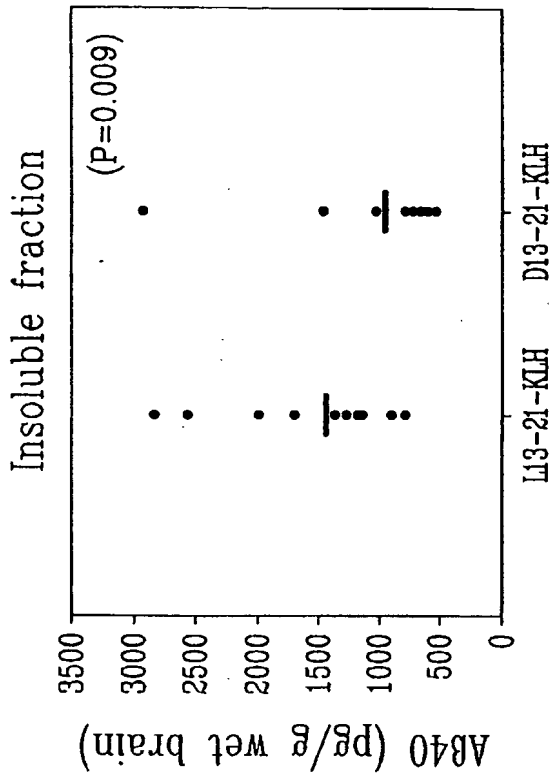

Soluble fraction
(P=0.008)
Aβ40 (pg/g wet brain)
900
800
700
600
500
400
300
200
100
L13-21-KLH
D13-21-KLH
Immunogen

FIG. 8A

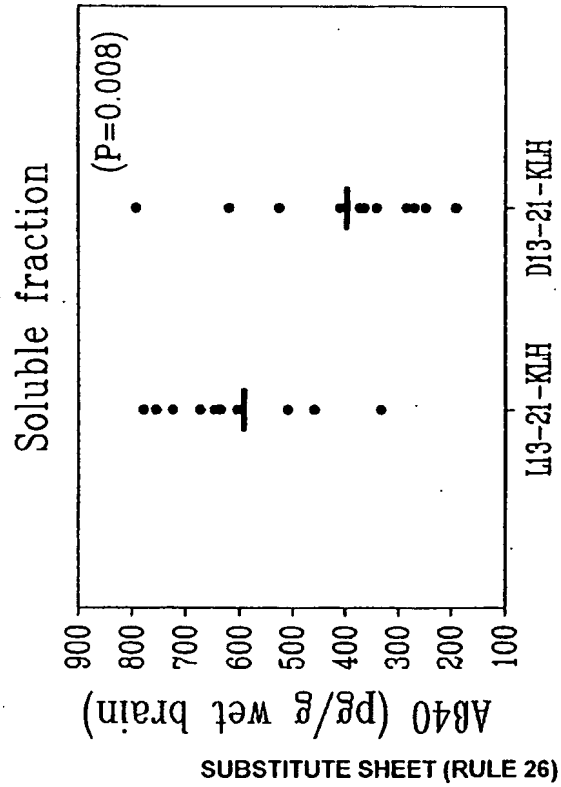

Insoluble fraction
(P=0.009)
Aβ40 (pg/g wet brain)
3500
3000
2500
2000
1500
1000
500
0
L13-21-KLH
D13-21-KLH
Immunogen

FIG. 8B

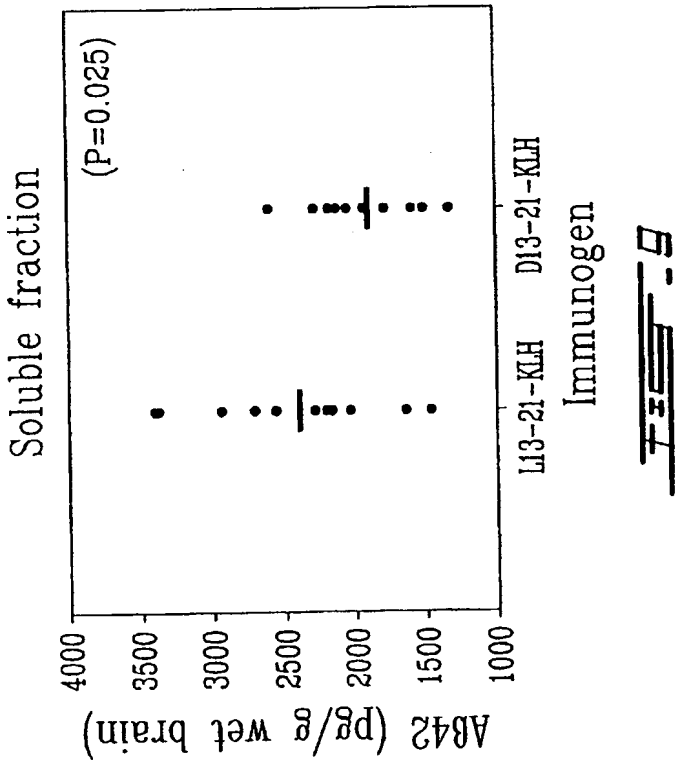

Soluble fraction
(P=0.025)
Aβ42 (pg/g wet brain)
4000
3500
3000
2500
2000
1500
1000
L13-21-KLH
D13-21-KLH
Immunogen

Fig. 5

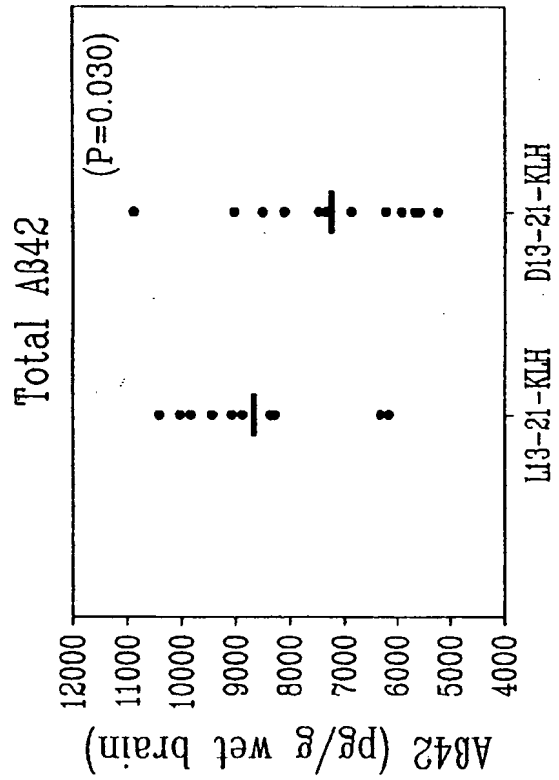


FIG. 10B

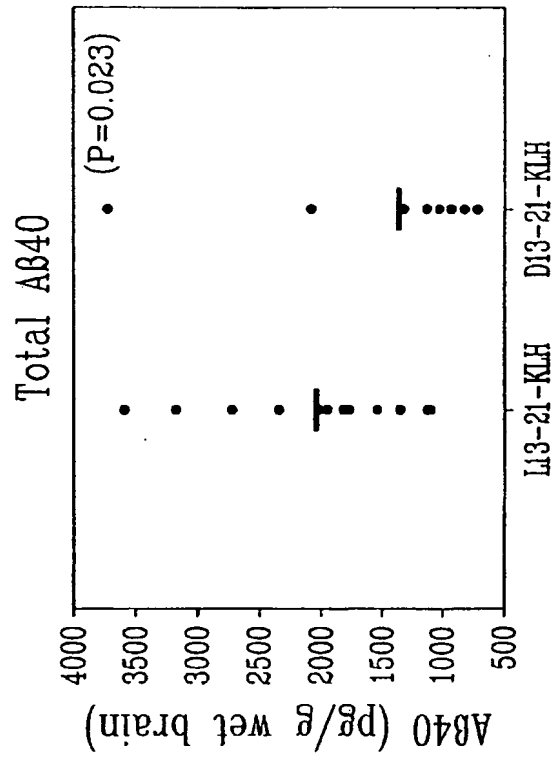


FIG. 10A